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MICROBIAL APPROACHES TO SUPPORT ANDEAN QUINOA PRODUCTION

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Plant Pathology and

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by

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Abstract

Quinoa, *Chenopodium quinoa*, is an Amaranthaceous seed crop, grown as a staple food and high-value export of the Andean region of South America. Research into quinoa production has increased due to the crop's ability to produce a highly nutritious seed under adverse environmental conditions. Quinoa production has increased greatly in South America in order to meet North American and European consumer demand. Increased production has led to the conversion of marginal land into quinoa fields and a subsequent decrease in sustainable production practices. Therefore, it is necessary to research sustainable methods of quinoa production. There has been substantial research into certain production methods, such as irrigation and improved quinoa lines, but there is little research into the use of microbes to enhance quinoa production.

Plant growth promoting *Bacillus* species were examined for their ability to colonize quinoa and to promote quinoa growth, but neither Bolivian isolates nor commercially available isolates were shown to enhance quinoa growth. Root colonization by *Bacillus* strains could only be reliably measured through the use of spontaneously generated rifampicin resistant mutants.

A subset of plant growth promoting bacteria, the phosphate solubilizing bacteria, were examined to determine if the tricalcium phosphate solubilizing phenotype was specific to certain *Bacillus* species. *Bacillus* populations isolated from Pennsylvanian *Chenopodium album*, Ecuadorean *C. quinoa*, and Bolivian *C. quinoa* were screened for the ability to solubilize tricalcium phosphate and this phenotype was paired with 16S sequence data to determine if there were species based patterns of tricalcium phosphate solubilization. Nearly all isolates of *B. megaterium* (100% of isolates) and *B. subtilis* (99%) groups were capable of tricalcium phosphate solubilization, whereas members of the *B. simplex* (51%) and *B. cereus* (39%) groups

varied greatly in their ability to solubilize tricalcium phosphate. This indicates the tricalcium phosphate solubilizing phenotype is ubiquitous in certain *Bacillus* species and these species could be targeted in screens for plant growth promoting bacteria. However, in assays in which the effects of a phosphate solubilizing *B. megaterium* were compared to a non-solubilizing *B. simplex* isolate, no differences were observed in plant growth or plant phosphorus content, so the role of the tricalcium phosphate solubilizing phenotype is still unclear.

The key disease of quinoa is downy mildew caused by *Peronospora variabilis* (formerly *Peronospora farinosa* f. sp. *chenopodii*). Seed-based detection methods using sequencing and PCR with specific primers were developed to detect seedborne oospores of *P. variabilis*. Thirty-two lots of imported, consumable quinoa seeds were screened using these methods and the pathogen was detected in 25 lots using the sequencing based method and 23 lots using the PCR with specific primers method. Therefore, seedborne oospores likely play a significant role in the spread of quinoa downy mildew and it is critical to develop management methods for seedborne oospores. Seedborne oospores are the likely source of initial inoculum for the introduction of *P. variabilis* to Pennsylvania. Phylogenetic analyses of the quinoa downy mildew pathogen from Ecuadorean and Bolivian quinoa samples showed that the pathogen that infects weedy *Chenopodium* species in Ecuador is *P. variabilis* (based on ITS sequences). Therefore, weedy *Chenopodium* species could harbor *P. variabilis* and further studies are required to determine if weedy *Chenopodium* can act as green bridges for *P. variabilis* between and during quinoa growing seasons.

To combine *Bacillus* and *P. variabilis* research together, biological control assays were performed to determine if *Bacillus* could reduce the severity of quinoa downy mildew infections. Both *Bacillus mycoides* BmJ and *Bacillus pumilus* GB34 appeared to reduce sporulation of *P.*

variabilis compared to an uninoculated control in two studies, but these results were non-significant.

Results of all these studies, along with protocols, were prepared for Ecuadorean and Bolivian collaborators, so that this research can be applied in key quinoa producing areas of South America. By identifying which *Bacillus* species groups solubilize tricalcium phosphate, collaborators can focus research efforts on these species groups in quinoa growth assays for plant growth promotion. Protocols for isolating and screening *Bacillus* isolates for plant growth promoting phenotypes, such as IAA and chitinase production, were developed to allow for consistent results amongst collaborators in the United States, Ecuador and Bolivia. Three new diseases of quinoa were reported in the United States, and through this research, simple, reliable methods for working with each pathogen were developed and shared with collaborators. Molecular methods to detect *P. variabilis* in quinoa seeds and plant tissue highlighted how quinoa seeds could potentially serve as sources of inoculum for *P. variabilis*. Finally, an extension program for rural Andean farmers in Tiraque Province, Bolivia was developed to teach farmers about integrated pest management of quinoa downy mildew.

Quinoa production could benefit greatly from research into how to manage both beneficial and pathogenic microbes. Incorporating beneficial microbes could help promote plant growth and reduce disease. Identifying sources of inoculum for *P. variabilis* and being able to rapidly detect *P. variabilis* in seeds and plant tissues will aid in development of integrated strategies to manage quinoa downy mildew. These microbial approaches could help support Andean quinoa production in years to come.

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Chapter 1

Introduction to Quinoa and Research Objectives

Quinoa, *Chenopodium quinoa*

Background

Quinoa, (*Chenopodium quinoa* Willd.) is a crop that is historically (46), economically (105) and culturally (80) important in its native Andean range. Historically, quinoa was a sacred crop of the Inca (33) and current consumption of quinoa, still culturally important, remains high in Bolivia and Peru (80; 105). Economically, Bolivian farmers' profit for quinoa has tripled between 2002 and 2009, a result of increased demand for quinoa by North American and European consumers (FAOSTAT, 2012).

Quinoa is valued for its complete protein content (102) as well as its ability to grow under harsh conditions (123). The conditions of its native Andean range include intense sunlight, drought (129), nutrient poor soils (17; 117), high saline soils (66) and frequent frosts. Because of its resilience, quinoa is considered to have potential as an important crop in the future of agriculture and interest in quinoa as an alternative cropping system has increased worldwide. NASA has determined that quinoa would be an excellent crop to use as a food source on long term space flights (108). Further, the United Nations Food and Agriculture Organization has supported efforts to increase production of quinoa (63).

Quinoa Production and Postharvest Processing

The top quinoa producing and exporting countries are Bolivia, Peru, and Ecuador (126). Aside from economic benefits, the increase in quinoa exports has had social impacts in exporting countries, such as dietary changes, with quinoa being substituted with cheaper rice and pasta products (105). In order to capitalize on the high value of quinoa, producers have often

shortened their rotations, removed pasture (that supported their llama food and dung source), which leads to depleted and impoverished soils.

The complete quinoa crop cycle takes 5-8 months (43) with quinoa planted during rainy periods in December or January in Ecuador (J. Ochoa, personal communication) and September through November (P. Backman, personal communication) in Bolivia. At maturity (May-June), the entire stalk is harvested, the quinoa plants are gathered in teepee-like clusters (seed-heads together) and allowed to dry for several weeks (43). After the stalks and seed-heads have dried, the seed-heads are threshed and the seeds dried further. The seeds are saved for local consumption or sold for further processing and export.

The quinoa postharvest process is quite laborious, due to the bitter flavored saponins that must be removed from the seeds but, as demand for the crop increases, postharvest processing becomes more mechanized (6; 43). Saponin removal can be performed on-site by small-scale farmers (6), but most often the grain is sold to a wholesaler that will process the seed in bulk (112). Small-scale farmers utilize washing, abrasion or small mills to remove saponins (6; 43). Large scale wholesalers can use one of two methods for saponin removal: mechanical abrasion or soaking/washing combined with agitation (43). The quinoa seed is either consumed domestically, as is common in Peru, or is exported, as is common in Bolivia.

Needs for Quinoa Crop Improvement

Quinoa shows a great deal of promise for worldwide cultivation, and as interest in the crop has increased, quinoa production has spread to Canada (120), India (16), Denmark (65), Poland (44) and the United States of America (32). Quinoa's ability to yield a nutritious seed under marginal conditions make it very desirable as more marginal land must be used for agriculture. Despite the potential benefits of increased quinoa production, there are also severe

limitations to the spread of quinoa production. Because quinoa is so well adapted to its native range, it can be difficult, if not impossible, to grow quinoa at lower elevations, where temperatures are warmer and rainfalls are higher. The spread of quinoa has been slow and fraught with difficulties, such as purported ancient curses (in the United States (121)) and high production costs and lack of productive varieties (in Canada (47)). In order to improve quinoa as a crop for worldwide deployment, locally adapted quinoa lines must be bred and sustainable disease, water and nutrient management practices must be established. Several areas of research that can potentially improve quinoa production are identified in the following discussion and include: breeding of enhanced varieties, use of irrigation, improved nutrient management and improved pest management.

Small-scale Andean farmers have traditionally used landraces of quinoa, but there is increased need for improved varieties, with traits such as disease resistance, as higher yields are demanded to meet consumer demand. However, there are few breeding programs in indigenous countries and breeding programs in first world countries have met with much difficulty. Upon patenting 40 Bolivian quinoa varieties to use in a breeding program (11), American researchers were sued by ANAPQUI (the Bolivian National Association of Quinoa Producers) and quinoa breeding efforts in the United States were effectively halted (130).

Despite some of the pitfalls of attempting to breed quinoa, researchers have developed effective tools that can be used by scientists in countries where quinoa breeding is more likely to be accepted. Genetic mapping of several lines of quinoa has been performed using simple sequence repeat markers (67) and gene linkage mapping has been performed by analyzing amplified fragment length polymorphism (AFLP) markers and random amplification of polymorphic DNA (RAPD) markers (85). An expressed sequence tag (EST) library for quinoa

was also developed (27). These genetic tools can be useful for expediting quinoa breeding. Quinoa can also be tissue cultured which would aid efforts to develop a transgenic quinoa variety (22). As quinoa production and breeding of locally adapted quinoa lines expands to other countries, understanding how different genotypes perform under different environmental conditions is essential (15). Extensive research has already been performed on how photoperiod (13), temperature (14) and different climates (15) affect how quinoa cultivars develop. Additionally, cytoplasmic male sterility can easily be manipulated in quinoa lines to develop reliably male sterile lines for hybridizations (136). However, even the best quinoa lines will yield poorly without adequate water and nutrient management.

Quinoa is drought tolerant but water management is still necessary to yield a good crop of quinoa seeds. There have been several extensive studies (52-55; 129) on how irrigation affects quinoa yields in the Bolivian altiplano. Quinoa is physiologically adapted to drought stress and researchers have found that under drought stress, quinoa's stomata rapidly close and quinoa has higher leaf water use efficiency (estimated by the ratio of leaf transpiration to photosynthetic rates) than other Andean crops (129). Quinoa yields could be improved if deficit irrigation (irrigation during key growth periods) is used during crop development periods that are drought sensitive (54), but irrigation can be expensive for low-input Andean farmers. In order to balance the price of irrigation with the slim benefits of irrigating a drought tolerant crop, researchers suggest limited irrigation of larger areas opposed to sustained irrigation of smaller areas (52). A combination of climatic and GIS data can be used to determine which regions of Bolivia would benefit the most from quinoa irrigation. However, quinoa deficit irrigation practices are not without drawbacks as they can lead to increased soil salinization which limits crop rotation options (55).

Andean farmers are low-input farmers, so cost-effective, simple nutrient management strategies are required for quinoa producers. Historically, the indigenous Andeans used llama dung (4; 135) and raised beds systems with *Azolla filiculoides*, an aquatic fern with symbiotic nitrogen fixing cyanobacteria, *Anabaena* (17), to combat the low soil nutrient levels of the Andean altiplano. However, maintaining an adequate supply of dung can be difficult, especially as grazing land is converted to agricultural land. The raised bed systems of the Inca and their ancestors may not be suitable for high production agriculture. One viable option for sustainable nutrient management may be the use of microbes that affect soil nutrient availability. Free-living nitrogen fixing bacteria and phosphate solubilizing microbes will likely play a key role in the expansion of sustainable agriculture in the Andean region of South America. Unlike most crops, quinoa is non-mycorrhizal (111; 127), so microbes that are able to increase phosphorus availability may enhance crop productivity.

Diseases and Pests of Quinoa

Quinoa Downy Mildew

Although quinoa is frequently used in plant pathology as a "universal" virus host, there is a lack of thorough knowledge about other diseases and pests of quinoa. Pests of quinoa can greatly reduce its yield (39) and control methods for these pests are often too expensive, ineffective, or underdeveloped.

The key disease of quinoa is downy mildew caused by *Peronospora variabilis* (formerly *Peronospora farinosa* f. sp. *chenopodii*) (26), an obligate, heterothallic (34) oomycete (35). Quinoa downy mildew is most severe under cool, humid conditions (35) and the pathogen over-seasons as oospores in seeds, leaves and soil (35). After over-seasoning, the oospore will germinate to form sporangia. The sporangia penetrate leaves directly and hyphae spread

intercellularly until sporangiophores are produced to initiate the secondary cycle of the disease (35). As disease progresses, chlorotic and necrotic spots are found on the leaves and grayish sporangia are visible on the underside of the leaf (35). Severe infections result in premature defoliation which leads to reduced yields and seed quality. Yield losses from downy mildew have been reported ranging from 33% to 100% (39; 40). Downy mildew of quinoa has been reported in most quinoa producing regions of the world including Canada (120), Denmark (38), India (76) and the United States (119).

Current management of quinoa downy mildew utilizes proper plant spacing, synthetic fungicides and resistant varieties (39). However, most quinoa growers cannot afford fungicides and it would be challenging to distribute resistant varieties (if developed) to the low resource, remote growers that need them. Extensive protocols for studying *P. variabilis* have been developed and released by the International Potato Center in collaboration with The Royal Veterinary and Agricultural University of Denmark (35). These protocols provide Andean researchers the groundwork to develop new management strategies for quinoa downy mildew.

Different races of *P. variabilis* have evolved, which can make disease management more difficult. Ochoa, et al. (93) determined that there were four virulence groups in Ecuadorean isolates of *P. variabilis* and there was specific resistance in quinoa lines to 3 of the 4 virulence groups. It should be noted that the quinoa line 'Faro' was susceptible to all four virulence groups of *P. variabilis* (93). Unfortunately, there is a lack of agreed upon screening methods for efficient and repeatable measurement of downy mildew severity in quinoa (36; 76), so reproducible testing of putative resistant quinoa lines can be difficult.

Molecular studies of *P. variabilis* have focused mainly on its taxonomy and have done little to support the concept of virulence groups of *P. variabilis*. Danielsen and Lübeck used

universally primed PCR to determine genetic differences amongst isolates of *P. variabilis* collected throughout the world. The authors found some minor differences between South American and Danish isolates of *P. variabilis.* However, the authors failed to analyze any isolates of *P. variabilis* from Bolivia, which is recognized as a center of origin for quinoa (37). If the techniques in this paper had been extended to more isolates, the authors might have been able to validate or enhance the findings of Ochoa, et al. (1999). An extensive survey of *P. variabilis* isolates from herbarium specimens of different *Chenopodium* species utilizing morphological and sequence analysis of the ITS region has demonstrated that there is great diversity between *Peronospora* species that infect *Chenopodium* species (25). Further morphological and ITS sequence analysis of *P. variabilis* isolates from Denmark and across the world indicate that *P. farinosa* f. sp. *chenopodii* should be reclassified as *P. variabilis* (26). However, samples of *P. variabilis* from Danish quinoa are again overrepresented in these studies and more extensive studies of genetic variability of Andean *P. variabilis* isolates are needed.

<u>Minor Diseases and other Pests of Quinoa</u>

There are several, less frequently reported diseases of quinoa, and compared to yield losses from quinoa downy minor, these diseases could be classified as minor diseases in quinoa production. *Phoma exigua* causes a stalk rot in quinoa, but only if the plant has been injured (39). Another disease of quinoa is a leafspot caused by *Ascochyta caulina* or *Aschochyta hyalospora* (18). Like *P. variabilis*, *A. caulina* and *A. hyalospora* can also be seedborne (18; 44).

When quinoa is grown outside of its native range, the most commonly reported diseases are damping off diseases caused by *Rhizoctonia solani* (39), *Sclerotium rolfsii* (10), *Pythium* species (45; 61), *Fusarium avenaceum*, and *Ascochyta caulina* (45). Damping-off diseases may

contribute to low rates of seedling germination and poor seedling vigor in quinoa production throughout the world.

There have been no reports of mycotoxin contamination in quinoa seed (94) or quinoa based food products (109). Several researchers have examined the fungal diversity of quinoa seeds. Commonly reported fungi found on quinoa seeds include *Ascochyta caulina*, *Alternaria* sp., *Fusarium* sp (44) *Aspergillus sp.*, *Penicillium* sp., *Mucor*, *Rhizopus*, *Phoma*, *Eurotium,* and *Ulocladium* (94). The saponins found in the quinoa seed coat have anti-fungal properties and these saponins, when alkali treated, have been demonstrated to reduce growth and inhibit conidia germination of *Botrytis cinerea* during in-vitro assays (118). The intensive post-harvest saponin removal process greatly reduces fungal contamination of quinoa seeds with seed percentage infection rates ranging from 4-88.5% of processed seeds being infected versus 100% of non-processed seeds being infected (94).

Quinoa is also affected by insect and nematode pests. In its native range, the major insect pests of quinoa are the quinoa moths (*Eurysacca melanocampta* and *E. quinoae*) and cutworms (100). Management of quinoa insect pests involves the usage of insecticides, parasitoids, and crop rotation (100). Thrips and aphids can also be problematic in quinoa production, especially in production of quinoa in the United States (in Colorado (32) and in Pennsylvania, personal observation). Aphids are reported pests in Denmark and Poland (100). Management of the most common quinoa feeding nematodes, *Naccobus aberrans* and *Thecavermiculatus andinus*, utilizes resistant quinoa lines and planting of *Chenopodium ambrosiodes* as an antagonistic crop due to the saponins it produces in its roots (49). Quinoa root extracts have been shown to paralyze juvenile root knot nematodes in greenhouse studies in Kenya (70).

Biological Control and Plant Growth Promotion by *Bacillus species*

Biological Control Utilizing *Bacillus* species

Bacillus species make ideal biological control agents for many reasons, including their ability to form shelf-stable and tank-mixable endospores (64), the ubiquity of *Bacillus* in the environment, the ability of *Bacillus* to induce host defenses (23), the ability of *Bacillus* to antagonize other microbes (83; 95) and the ability of *Bacillus* to enhance nutrient availability to plants (1; 60; 113). The role of *Bacillus* endophytes in agriculture has been extensively examined (7) and *Bacillus* endophytes have been studied as biological control agents in many crops including corn (86), cotton (73; 81), cacao (88), and peanut (124).

Biological control agents can act on one or several plant tissue types simultaneously. Root populations of microbes can affect foliar populations of microbes, which is shown in the control of bean rust *(Uromyces appendiculatus*) by several root applied *Pseudomonas* species in common bean (2). The alternative is also true, foliar microbial populations can affect root microbial populations, as demonstrated by Rudrappa, et al. (107) in which *Arabidopsis thaliana* plants that were foliarly inoculated with *Pseudomonas syringae* produced and exuded malic acid from their roots and this carbon source allowed for increased root colonization by *Bacillus subtilis*. Microbial populations can also interact within the same area of the plant, such as the phyllosphere. The microbial ecology of the leaf is quite complicated, with many microbe-microbe and plant-microbe interactions occurring simultaneously (77), thus application of *Bacillus* to leaves as biological control agents of foliar diseases of quinoa, such as downy mildew, may also be possible.

The use of *Bacillus* to manage diseases of quinoa has not been studied, but there are related studies that examine the ability of *Bacillus* to control diseases related to those in quinoa

and in crops related to quinoa. *Bacillus* isolates have been examined for their ability to reduce downy mildew severity (caused by *Sclerospora graminicola*) and promote plant growth in pearl millet (*Pennisetum glaucum*) and formulations containing either GB122 and GB34 or GB122 and T4 (*Bacillus subtilis* and *Bacillus pumilus*, respectively, for each pair) slightly reduced severity of downy mildew in greenhouse and field trials (99). There are few other studies of the biological control of downy mildews, but the use of *Pseudomonas fluorescens* (125) or bacterial derived biosurfactants (116) have been studied. *Bacillus mycoides* BacJ is capable of reducing severity of *Cercospora* leaf spot in sugar beet (9), but the usefulness of this *B. mycoides* isolate has not been examined in other Amaranthaceous hosts, such as quinoa.

Plant Growth Promotion by *Bacillus* species

Bacillus species can promote plant growth through several mechanisms. As previously discussed, *Bacilli* antagonize pathogens or induce host defenses to promote plant growth through disease reduction. *Bacilli* can stimulate existing plant-microbe symbioses, such as rhizobial (124) or mycorrhizal (122) relationships, that enhance nutrient uptake by the plant. *Bacilli* affect plant growth through the production of plant hormones such as auxins (97), cytokinins (5) and gibberellic acid (59) and by affecting hormone levels, such as by producing ACC deaminase (56). *Bacilli* may provide nutrients to plants either through nitrogen fixation (1; 60) or phosphate solubilization (41).

Plant Growth Promotion by Phosphate Solubilizing Bacteria

Phosphate solubilizing bacteria (PSB) are a subset of plant growth promoting bacteria. It is thought that these bacteria can provide plants with phosphorus from insoluble forms of phosphate in the soil (104). Many phosphate solubilizing bacteria have other characteristics of plant growth promoting bacteria, such as the ability to antagonize pathogens and produce plant

growth hormones (41; 104). Therefore, it can be difficult to determine how much the phosphate solubilizing phenotype actually benefits the plant.

Research Objectives

Previous quinoa research has not focused on microbes associated with quinoa. Research for this thesis focused on *Chenopodium* associated *Bacillus* species and disease caused by *Peronospora variabilis*. The objectives of this master's research were as follows:

1. To determine if *Bacillus* species can colonize and enhance the growth of quinoa
2. To determine which *Chenopodium* associated *Bacillus* species are capable of phosphate solubilization
3. To examine the phylogeny of *Peronospora variabilis* and develop a rapid method for detecting *P. variabilis* in quinoa seed and plant tissue
4. To work with collaborators in Ecuador and Bolivia to develop protocols for and determine future needs for quinoa-microbe interaction research

Chapter 2

Colonization of *Chenopodium quinoa* by *Bacillus* species and Effects on Quinoa Growth

Introduction

Plant growth promoting bacteria (PGPB) or rhizobacteria (if root associated, PGPR) are plant associated bacteria that have been demonstrated to enhance plants growth and reduce disease in a wide range of hosts and growing conditions (28).

Colonization of plant tissues by biological control agents (BCA) has been demonstrated to be an effective means of disease reduction (110) and plant growth promotion. By colonizing tissues normally affected by pathogenic microbes, biological control agents can reduce disease by antagonism (75), inducing host defenses (9), by resource competition (79), or physical exclusion of the pathogen from the host (90). Biological control agents can have effects even at low population levels (101), so high levels of colonization may not always be a prerequisite for effective plant growth promotion.

The ability to measure the colonization abilities of different PGPB or BCAs is essential for selecting the best competitors for further study and possible development into commercial products. If the bacterium being studied does not have selectable traits for isolation from host tissue, such as tolerance to low pH and high salinity (124), it becomes necessary to introduce a selectable marker into the strain being studied (72). Selectable markers, most commonly antibiotic resistance, can be introduced via resistance plasmids, but it is often easier to select for spontaneous antibiotic resistant mutants (133).

This series of experiments sought to determine the effect of *Bacillus* isolates from Bolivian quinoa and commercially available PGPR strains on the growth of *Chenopodium quinoa*. The objectives of these experiments were twofold: i) to determine the level of colonization by each *Bacillus* strain and ii) to determine if there are any measureable effects on each strain on quinoa growth. A series of growth chamber and field trials were undertaken to address these objectives.

Materials and Methods

Bacillus Strains

In this study, five *Bacillus* strains provided by collaborators working for Fundación PROINPA (Foundation for Promotion and Investigation of Andean Produce) as the investigators identified these strains as capable of promoting quinoa growth. These strains were isolated from quinoa roots (H2.1, H2.2, H2.3, and H2.5) or quinoa leaves (H2.4) from Tiraque Province, Bolivia. Strains were identified using partial 16S ribosomal RNA sequencing and included the following isolates: H2.1 *Bacillus megaterium*, H2.2 *B. pumilus*, H2.3 *Bacillus cereus* group, H2.4 *Bacillus atrophaeus*, and H2.5 *Bacillus pumilus*. Bolivian strains were compared to several commercially available *Bacillus* strains used for biological control and plant growth promotion, including GB34 *B. pumilus*, GB99 *Bacillus amyloliquefaciens*, GB122 *Bacillus subtilis* and FZB24 *B. amyloliquefaciens*. Strains were stored in 20% glycerol in liquid nitrogen for long-term storage.

Bacterial and Plant Growth Conditions and Inoculation Methods

Isolates were streaked in pure culture on trypticase soy agar (TSA) and a single colony was picked and inoculated into 100 mL of trypticase soy broth (TSB, EMD Chemicals).

Inoculated flasks were incubated for 2 weeks at 30° C with rotation of 150 rpm. This long growth period was to exhaust the media and induce endospore formation. After two weeks, cultures were transferred to sterile 50 mL centrifuge tubes and centrifuged at 5,000 rpm for 15 minutes and the culture supernatants were discarded. The pellet was suspended in 25 mL sterile milliQ water and spun again at 5,000 rpm for 15 minutes and the supernatant was discarded. This washing process was repeated two more times to remove germination inhibitors and bacterial waste products produced during culture growth. Cleaned pellets were suspended in 0.7% sodium chloride and viable cell counts were determined by plating dilutions onto trypticase soy agar using the Autoplate 4000 spiral plater (Spiral Biotech Inc., Norwood, MA). Cultures were adjusted to 10^6 CFU/mL unless noted otherwise (specific concentrations are listed in each experiment) and these suspensions served as the inoculum for plant inoculations.

For each experiment, quinoa seeds were sown ¼ inch deep in pre-moistened potting media and 1 mL of bacterial or control suspension was delivered onto each seed. Seeds were covered with potting media. Hoagland's solution (either with or without added phosphate) was used to fertilize plants on a once weekly basis. Plants were grown in growth chambers (Trial 1 used an EGC chamber, Trials 2, 3 and 4 used a Conviron growth chamber) with a 12 hour day/night cycle and with day temperatures of 24° C and night temperatures of 21-22° C with 65% relative humidity. Lights were set to the maximum brightness setting.

Colonization Trial 1

In this trial, only the Bolivian isolates were tested along with a sterile saline control for a total of 6 treatments with 6 replications arranged in a randomized complete block in the growth chamber. Plants were grown in unpasteurized Fafard 2 (Conrad Fafard Inc., Agawam, MA)

potting mix in 4 inch pots. The following bacterial concentrations were used in this trial: H2.1 (log_{10} 5.6), H2.2 (log_{10} 6.3), H2.3 (log_{10} 6.1); H2.4 (log_{10} 6.3), and H2.5 (log_{10} 6.7).

Plants were harvested 42 days after sowing and inoculation. Above ground shoot mass (both fresh and dry), plant height and number of internodes were recorded. Roots were collected by shaking and manually brushing off excess potting mix until only the rhizospheric soil remained. Roots were shaken in sterile distilled water and blotted on sterile paper towels to dry. A composite of one gram of the fresh root was weighed and placed into a sterile sample bag. Samples were washed in 25 mL sterile water in a stomacher blender (Bagmixer 100, Intersciences, Saint Nom, France) for 60 seconds (speed 7), the water was poured into sterile 15 mL tubes for root surface populations, and was followed by a 30 second wash in 25 of additional sterile water. Roots were blotted dry on sterile paper towels and transferred to a sterile sample bag and macerated with a hammer until tissue was completely disrupted. 25 mL of sterile 0.7% saline was added to the bag and the samples were homogenized in stomacher blender for 60 seconds at maximum speed. A manual trituration and a stomacher based method of bacterial recovery was chosen based on previous studies with *Bacillus* species (73).

Colonization Trial 2

Due to high background levels of *Bacillus* colonists in non-inoculated control plants in trial 1, a sterile sand media was used in trial 2. Additionally, 1 g of tricalcium phosphate ($Ca_3(PO4)_2$, Sigma Aldrich, St. Louis, MO) was added to 150 mL of tamped silica sand (Quikrete, Atlanta, GA) in 250 mL flasks. Flasks were autoclaved for 1 hour, allowed to sit for 24 hours, and autoclaved for one additional hour. Conetainers (Leach, SC10 Super, 164 mL volume, 3.8 cm diameter, 21 cm depth, bleached to decrease microbial contamination) were used

in this trial. The following nine treatments were used in trial 2 (followed by concentration of initial inoculum): H2.1 (log_{10} 5.7), H2.2 (log_{10} 6.1), H2.3 (log_{10} 6.9), H2.4 (log_{10} 6.9), H2.5 (log_{10} 6.7), GB34 (log_{10} 6.7), GB122 (log_{10} 6.7), FZB24 (log_{10} 6.5), and no bacteria (sterile 0.7% NaCl).

Plants were harvested 65 days after planting and shoot mass (both fresh and dry) and plant height were recorded. Excess potting mix was shaken and brushed from the roots so that only rhizospheric sand remained. Roots were dried in the growth chamber for 3 days to induce endospore formation by *Bacillus*. Root dry mass was recorded and roots were placed in a sterile sample bag. Roots were mechanically disrupted by smashing with a hammer, 5 mL of sterile 0.1 M phosphate buffer was added and a stomacher blender was used to homogenize the samples (60 seconds at maximum speed). These two methods, mechanical disruption and stomacher disruption, should have provided good recovery of *Bacillus* populations (73). Liquid from the homogenized sample was pipetted into sterile, 1.5 mL tubes and diluted for enumeration. Diluted samples were plated on TSA using a spiral plater. Plates were incubated at 30° C for 24-48 hours.

Colonization Trial 3

Due to the slow growth and stunting of plants in sand, a peat based potting mix (Metro Mix 360, Sun Gro Horticulture, Vancouver, Canada) in Conetainers was used in trial 3. One g of tricalcium phosphate was added to 150 mL of potting mix and the mix was sterilized by two sequential autoclavings as described in trial 2. The treatments used in trials 3 (followed by inoculum concentration) were as follows: H2.1 (log_{10} 6.1), H2.2 (log_{10} 7.0), H2.3 (log_{10} 6.1),

H2.4 ($\log_{10}$ 6.9), H2.5 ($\log_{10}$ 7.1), GB34 ($\log_{10}$ 6.8), GB99 ($\log_{10}$ 6.3), GB122 ($\log_{10}$ 6.7), FZB24 ($\log_{10}$ 6.6) and no bacteria (sterile saline).

Plants were harvested 43 days after planting and shoot mass (both fresh and dry) and plant height were collected. Excess potting mix was shaken and brushed from the roots so that only rhizospheric potting mix remained and roots were dried in the growth chamber for 3 days to induce *Bacillus* sporulation. Root dry mass was recorded and roots were placed in a sterile sample bag. Roots were macerated with a hammer, 5 mL of sterile 0.1 M phosphate buffer was added and samples were homogenized in a stomacher blender (60 seconds at maximum speed). The homogenized samples were placed in sterile microfuge tubes and plated onto TSA using a spiral plater. After initial plating, the tube was heated at 75° C for 15 minutes to kill off vegetative cells. Heated samples were plated again to determine the number of endospores per sample. Plates were incubated for 24 hours at 30° C.

Rifampicin Resistant Mutant Colonization Trial

Selection of Spontaneous Rifampicin Resistant Mutants

Rifampicin was chosen as a selection marker for this study due to its broad spectrum antibiotic properties and its use in previous colonization studies (8). Rifampicin resistant mutants were selected for by using modifications of procedures used by previous researchers (21; 62; 133). Bolivian isolates and commercial *Bacillus* isolates were inoculated into 50 mL of TSB and incubated for 48 hours at 30° C with shaking. Cultures were pelleted through centrifugation and suspended in 5 mL of sterile water. 100 µL of each culture suspension was plated on TSA (4 plates per isolate) amended with 100 µg/mL of rifampicin. Inoculated petri dishes were placed in a dark box and incubated at 30° C for 48 hours. After incubation, three

colonies with wild type morphology were selected from plates of each isolate (except for H2.1 and H2.3, which had only 1 spontaneous mutant). Selected spontaneous rifampicin resistant mutants were sequentially passaged five times on TSA amended with 50 µg/mL rifampicin. Sequential passaging of the isolates ensured the stability of the rifampicin resistance mutation. For production of endospores of rifampicin resistant mutants for colonization trials, the same procedure as described above was used, except that TSB was amended with 50 µg/mL rifampicin and cultures were incubated in the dark.

Growth Conditions and Isolation of Rif^R Mutants

MetroMix 360 (autoclaved 30 minutes, no tricalcium phosphate added (to reduce variables)) was used in the Rif^R trial and conetainers were used as in the two previous trials. The following treatments were used in the Rif^R colonization trial: H2.1R1 (log_{10} 4.3), H2.2R3 (log_{10} 5.8), H2.3R1 (log_{10} 6.0), H2.4R3 (log_{10} 6.1), H2.5R2 (log_{10} 6.2), GB34R3 (log_{10} 5.7), GB99R1 (log_{10} 6.1), GB122R2 (log_{10} 4.7), FZB24R1 (log_{10} 6.0) and no bacteria (sterile saline).

Plants were harvested 28-29 days after planting and data on fresh and dry biomass and plant height were collected. Excess potting mix was shaken and brushed from the roots so that only rhizospheric potting mix remained. Roots were placed in a sterile sample bag, macerated with a hammer and 5 mL of 0.1 M phosphate buffer was added. Samples were mixed in a stomacher at maximum speed for 1 minute. Samples were dilution plated by a spiral plater onto TSA amended with rifampicin. Plates were incubated at 30° for 24-48 hours.

Field Trial

The following commercially available *Bacillus* isolates (followed by inoculum concentration) were used as treatments in the 2011 field trials at Rock Springs: GB34 (log_{10} 6.1),

GB122 (log_{10} 6.3), GB99 (log_{10} 6.84), and no bacteria (sterile saline). Three quinoa seed lines were used in this experiment: 'Faro,' AlterEco White (White), AlterEco Red (Red). The twelve treatments in this experiment are as follows: Faro with GB34, Faro with GB99, Faro with GB122, Faro with no bacteria, White with GB34, White with GB99, White with GB122, White with no bacteria, Red with GB34, Red with GB99, Red with GB122, and Red with no bacteria. Seeds were planted in 67 unit plug trays containing MetoMix 360, with each plug holding a volume of approximately 55 mL (32 mm diameter, 90 mm deep). One mL of each bacterial or control suspension was applied to seeds in the plugs at the time of planting. Quinoa seedlings were started in the greenhouse for two weeks, after which time flats were moved to Rock Springs to harden off seedlings.

Approximately one-month (28 day) old seedlings were transplanted into the research field by hand. A randomized complete block design was used in this experiment with 6 replicates of each treatment. Plants were spaced 6 inches apart and rows were spaced 8 inches apart. 36 plants (3 rows of 12) of 'Faro' were planted into each appropriate plot and 8-12 plants (due to poor germination and seedling survival) of White and Red quinoa were planted into each appropriate plot.

'Faro' plant tissue was collected for nutrient analysis at the initiation of seed head formation (8/12/11). The two youngest, but fully expanded leaves were collected from the main stalk of each plant of 'Faro' treatments. Foliar samples were bagged and dried at 70° C for 36 hours. Samples were frozen until ground with a mortar and pestle in sterile sample bags. Samples were submitted to the Penn State Agricultural Analytical Lab for tissue nutrient analysis.

Entire 'Faro' quinoa plants were harvested from field plots 103 days after planting. Due to poor growth of White and Red quinoa and high mortality during the growing season, these plants were not harvested. For 'Faro,' roots were cut at the soil line and dried in the greenhouse for 3 days, then moved to storage at 2° C for further analysis. The aboveground portion of the plant was dried for 48 hours at 65° C. Data on plant height, the number of side branches, and per plant dry biomass were collected from the harvested plants.

To determine root colonization, 5 roots were selected from each treatment, excess soil was sprayed off with compressed air, and 1 gram of each taproot was placed in a sterile sample bag. If the taproot did not weight 1 gram, the largest lateral root was included. The pooled 5 g of root were placed in a bleached Waring blender and ground in 500 mL of sterile milliQ water at high speed for 3 minutes. Ground samples were filtered through two layers of sterile cheesecloth into a sterile 500 mL flask. Samples were shaken thoroughly and 3 mL of sample was pulled from the tube and placed into 3 - 1.5 mL microfuge tubes. Thousand-fold diluted samples were spiral-plated in triplicate on TSA. The original sample tubes were heated at 75° C for 15 minutes and the 100 fold dilution was plated on TSA and V8 agar plates in triplicate. Plates were incubated at 30°C for 24 hours.

Results

<u>Trial 1</u>

A one way analysis of variance was used to analyze the effects of treatment on colonization, fresh biomass, dry biomass, shoot length (Figure 2.1) and the number of internodes. Neither colonization ($p=0.146$), number of internodes ($p=0.103$), nor shoot length ($p=0.517$) showed any significant differences due to treatment. Fresh biomass ($p=0.018$) and dry biomass

(p=0.007) were significantly different based on a one way ANOVA (α=0.05) with treatment as the factor. For fresh mass, H2.1 significantly increased the fresh biomass compared to H2.3, based on Tukey's HSD. For dry mass, isolates H2.1 and H2.2 significantly increased shoot mass compared to H2.3, based on Tukey's HSD. However, neither H2.1 nor H2.2 had significantly higher levels of fresh biomass or dry biomass compared to the non-inoculated control. Based on these results, including that the non-inoculated control had equivalent levels of colonization, no conclusions could be drawn from this experiment.

Trial 2

Similar to trial 1, no evidence for plant growth promotion or effective colonization was discovered during trial 2. Based on one way ANOVA (α=0.05), *Bacillus* treatment had no significant effect (Figure 2.2) on colonization (p=0.643), shoot length (p=0.302), fresh biomass (p=0.444), or dry biomass (p=0.301). Again, colonization levels of non-inoculated controls plants were equivalent to colonization levels of inoculated plants.

Trial 3

In trial 3, no solid evidence was gathered on the effects of *Bacillus* on plant growth promotion in quinoa. Based on one way ANOVA (α=0.05), there was no significant difference (Figure 2.3) due to treatment for colonization (unpasteurized, p=0.406, pasteurized, p=0.0904), fresh biomass (p=0.885), dry biomass (p=0.714), or shoot length (p=0.728). Again, the findings of trial 3 are similar to trials 1 and 2, because no conclusions about colonization or plant growth promotion can be drawn.

Rifampicin Resistant Mutant Trial

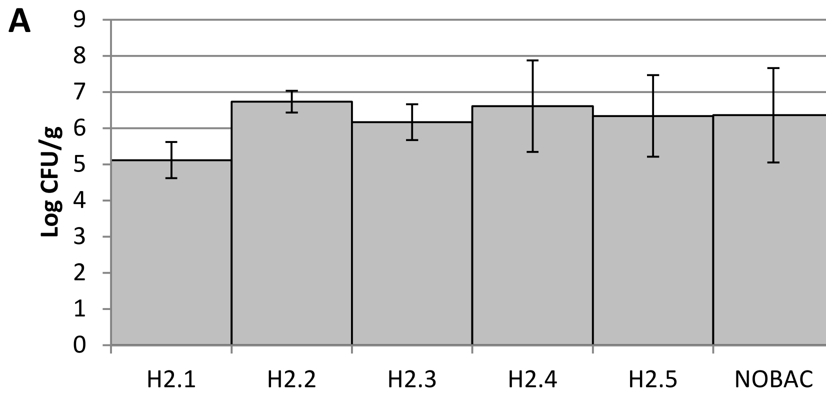
Based on one way ANOVA (α=0.05), bacterial treatment accounted for a significant difference (Figure 2.4) in colonization (p=0.000), but no significant differences were observed for fresh mass (p=0.572), dry mass (p=0.596) or shoot length (p=0.882). In the rifampicin resistance trials, significantly different levels of colonization were achieved for all bacterial treatments, excluding H2.1, compared to the uninoculated control. However, again no evidence for plant growth promotion was seen based on the results of the rifampicin resistance trial.

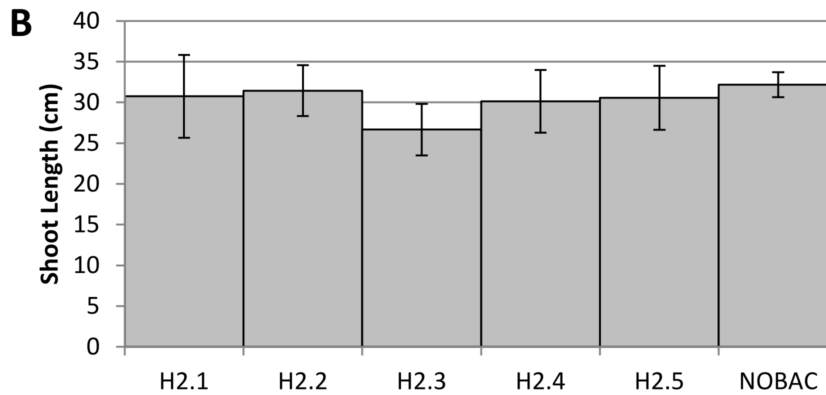
Field Trial

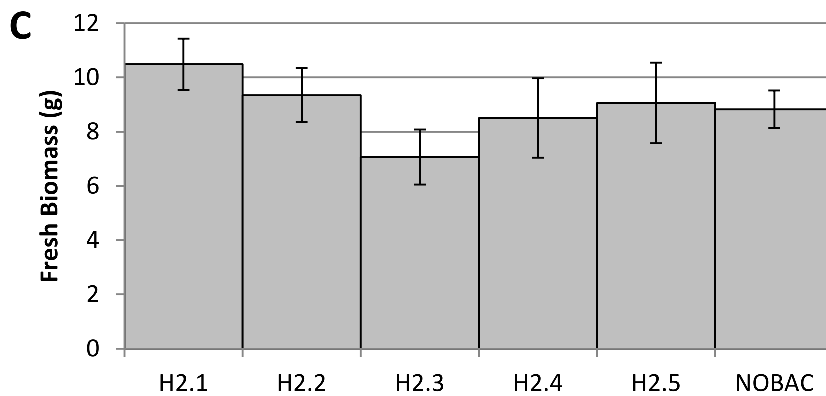
Although, no evidence for plant growth promotion was observed in growth chamber trials, field trials could provide some evidence for plant growth promotion. Colonization was not significant (Figure 2.5) for either unheated (p=0.806) or heated (p=0.66) measures of bacterial CFU/g root tissue. Seed weight was also not significant (p=0.529). Neither biomass (p=0.808), side branching (p=0.510), nor plant height (p=0.561) was significant. There were no significant differences between treatments for any of the plant tissue nutrient levels (based on one way ANOVA (α=0.05) N, p=0.99; P, p=0.89; K, p=0.95; Ca, p=0.22; Mg, p=0.15; S, p=0.47; Mn, p=0.11; Fe, p=0.51; Cu, p=0.16; B, p=0.53; Al, p=0.7; Zn, p=0.57; Na, p=0.81).

No plant growth promotion by disease reduction was observed. By season's end, each plot of 'Faro' had high incidence of quinoa downy mildew (Appendix A), *Passalora* leafspot (Appendix B) and *Ascochyta* leafspot (Appendix C).

In summary, plant growth promotion from *Bacillus* treatments was not observed in any colonization or field trials. Reliable colonization levels were only obtained in the trial that used rifampicin resistant mutants.







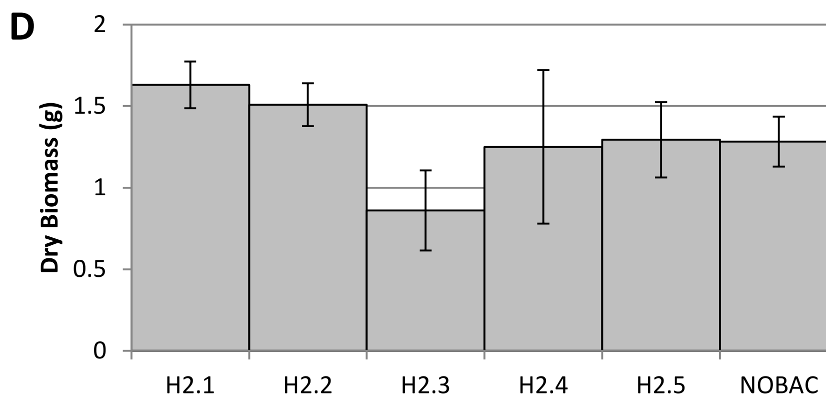


Figure 2-1 Results of trial 1 of *Bacillus* colonization of quinoa 'Faro';

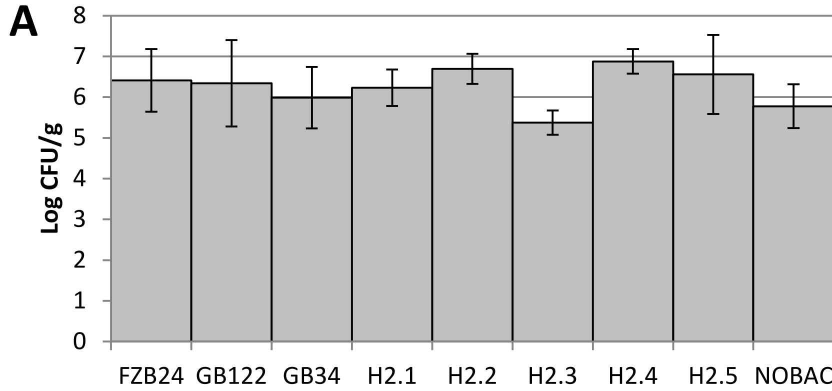
A) Root colonization of each *Bacillus* isolate expressed as CFU/g of fresh root tissue, NOBAC is the non-inoculated control;

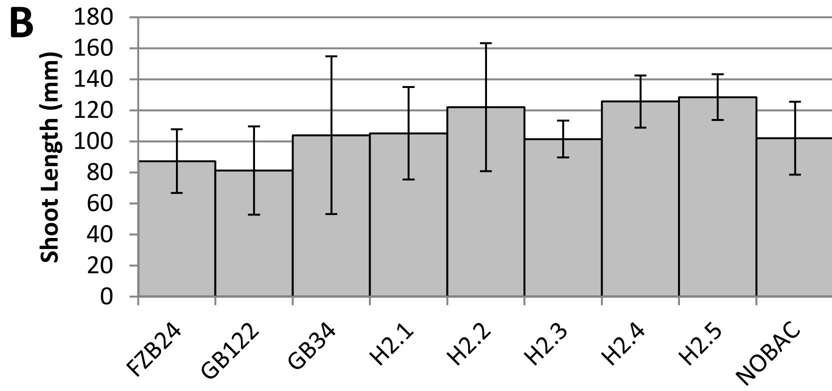
B) Mean shoot length for each *Bacillus* treatment;

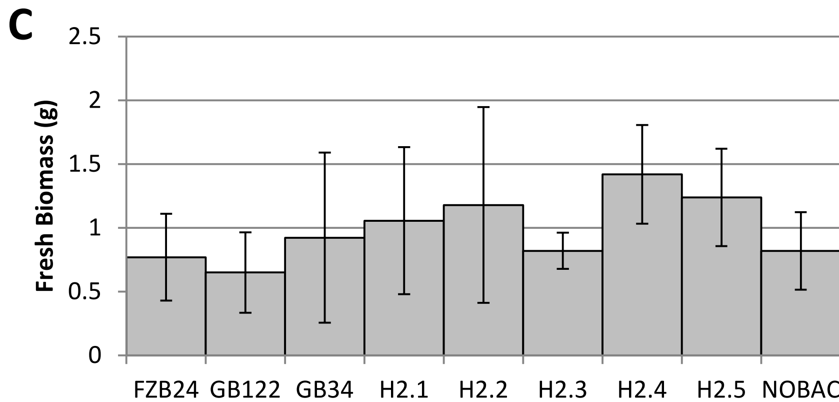
C) Mean fresh aboveground biomass for each *Bacillus* treatment;

D) Mean dry aboveground biomass for each *Bacillus* treatment

Error bars are standard deviation.







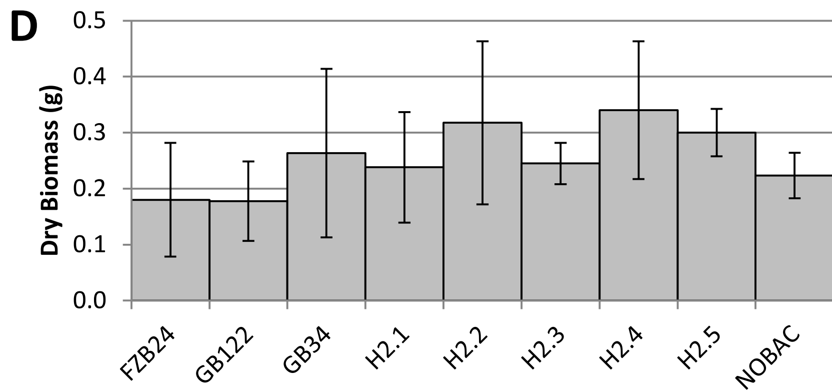


Figure 2-2 Results of trial 2 of *Bacillus* colonization of quinoa 'Faro';

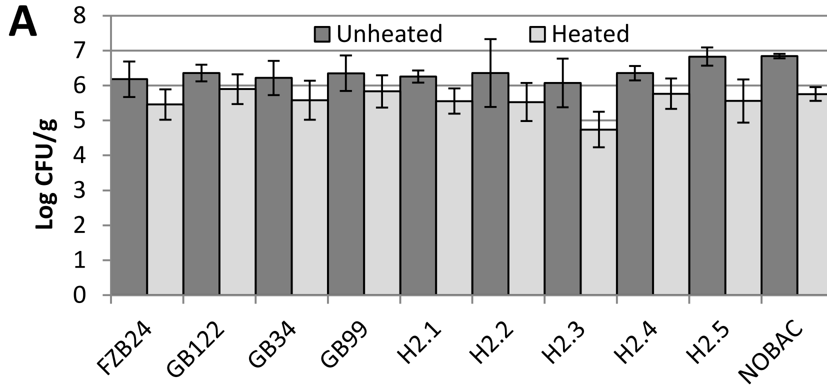
A) Root colonization of each *Bacillus* isolate expressed as CFU/g of dry root tissue, NOBAC is the non-inoculated control;

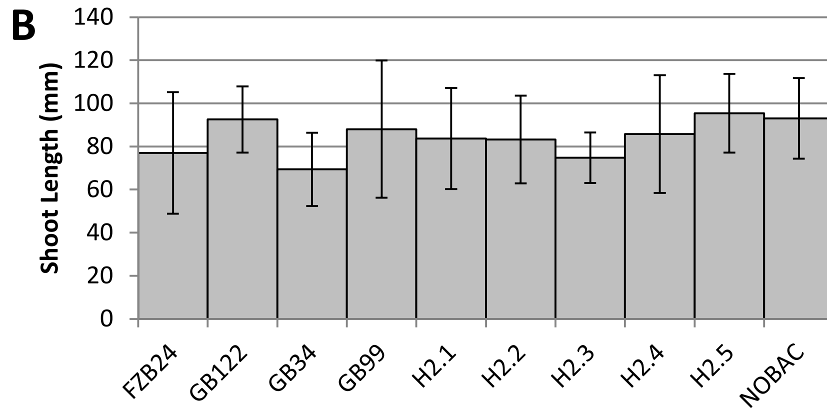
B) Mean shoot length for each *Bacillus* treatment;

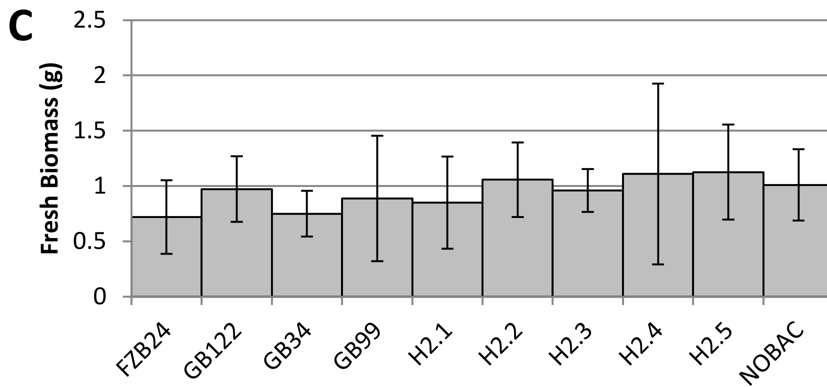
C) Mean fresh above ground biomass for each *Bacillus* treatment;

D) Mean dry aboveground biomass for each *Bacillus* treatment

Error bars are standard deviation.







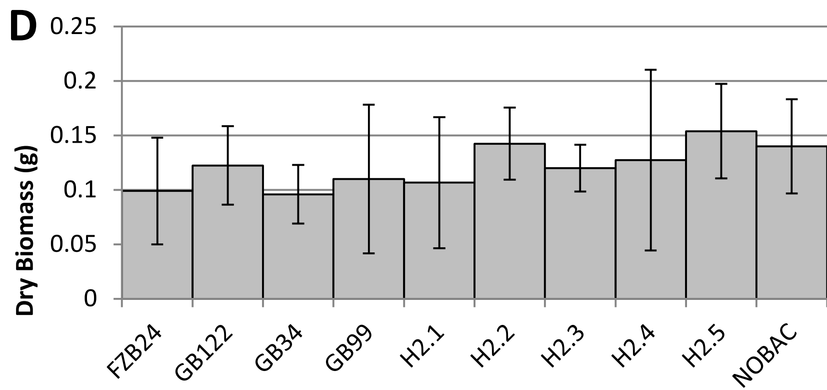


Figure 2-3 Results of trial 3 of *Bacillus* colonization of quinoa 'Faro';

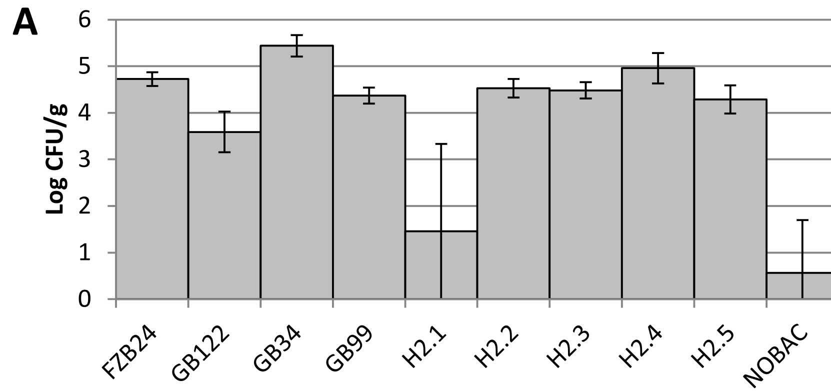
A) Root colonization of each *Bacillus* isolate, showing both total bacteria (unheated) and pasteurized endospores (heated), expressed as CFU/g of dry root tissue, NOBAC is the non-inoculated control;

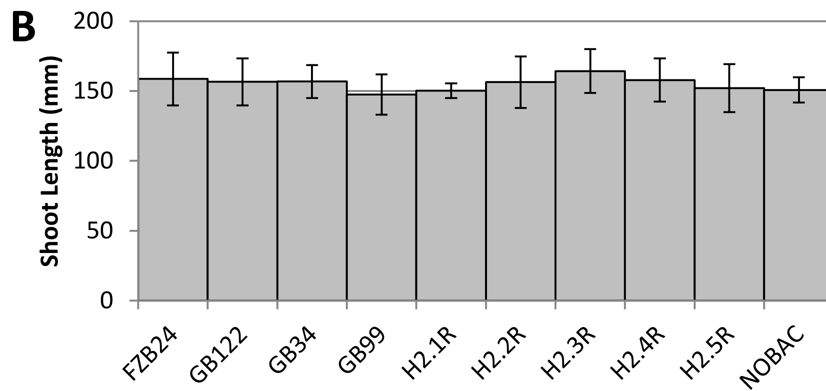
B) Mean shoot length for each *Bacillus* treatment;

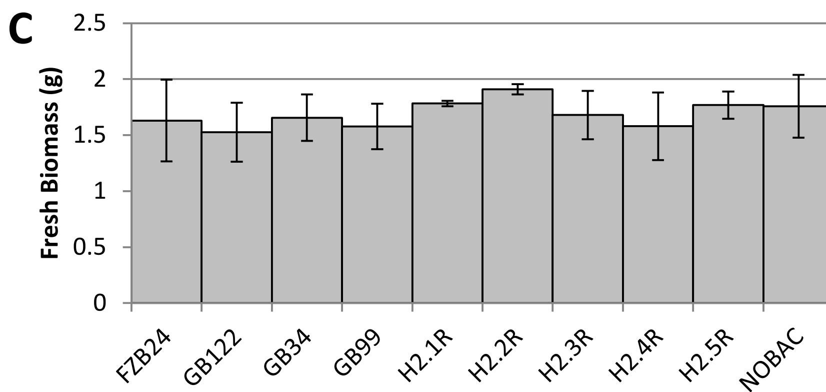
C) Mean fresh above ground biomass for each *Bacillus* treatment;

D) Mean dry aboveground biomass for each *Bacillus* treatment

Error bars are standard deviation.







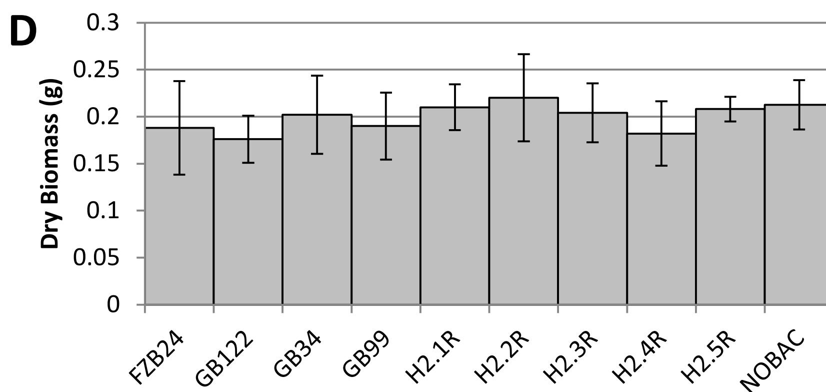


Figure 2-4 Results of Rifampicin resistant *Bacillus* colonization of quinoa 'Faro';

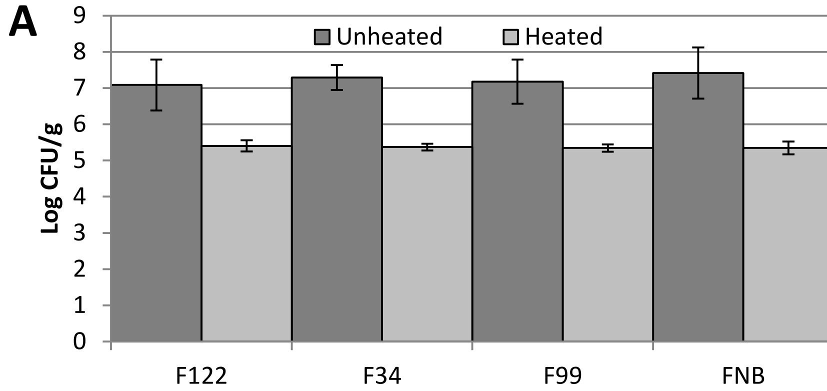
A) Root colonization of each Rif^R *Bacillus* isolate expressed as CFU/g of fresh root tissue, NOBAC is the non-inoculated control;

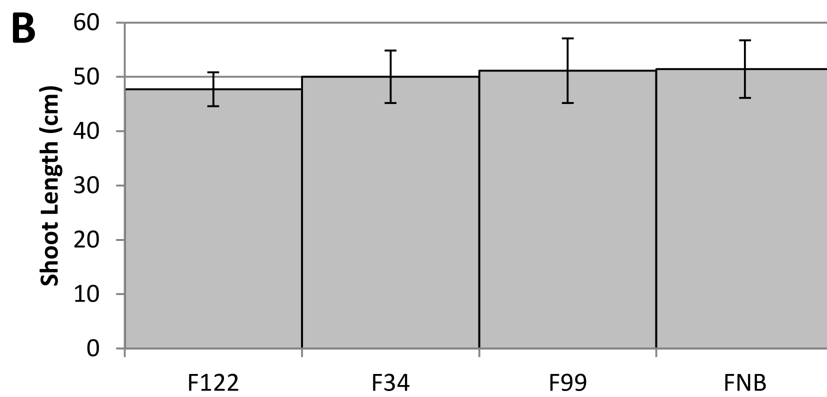
B) Mean shoot length for each Rif^R *Bacillus* treatment;

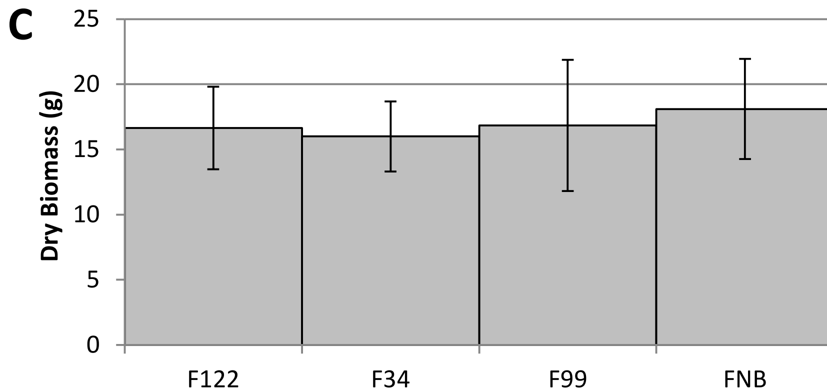
C) Mean fresh above ground biomass for each Rif^R *Bacillus* treatment;

D) Mean dry aboveground biomass for each Rif^R *Bacillus* treatment

Error bars are standard deviation.







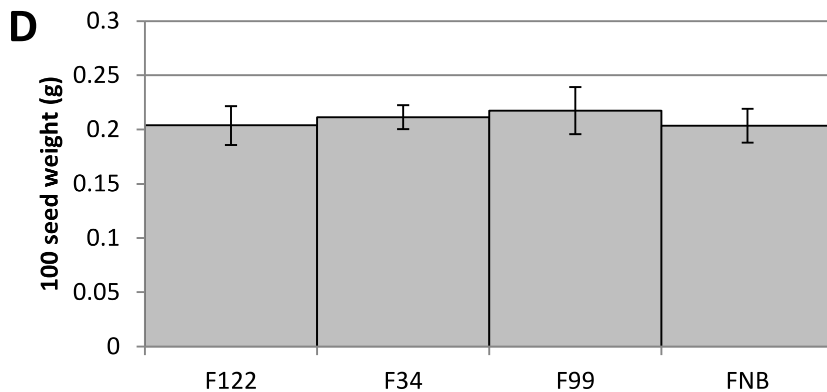


Figure 2-5 Results of field trials of commercially available *Bacillus* colonization of quinoa, F122 is 'Faro' treated with GB122, F34 is 'Faro' treated with GB34, F99 is 'Faro' treated with GB99 and FNB is 'Faro' treated with no bacteria;

A) Root colonization of each *Bacillus* isolate expressed as CFU/g of dry root tissue, showing both total bacteria (unheated) and pasteurized endospores (heated) NOBAC is the non-inoculated control;

B) Mean shoot length for each *Bacillus* treatment;

C) Mean per plant dry above ground biomass for each *Bacillus* treatment;

D) Mean 100-seed weight for each *Bacillus* treatment

Error bars are standard deviation.

Discussion

Plant growth promotion was not observed in any of these trials and this may be due to several factors. First, the cultivar of quinoa used in these trials, 'Faro,' may have been too genetically diverse to provide consistent phenotypes. There is evidence that quinoa lines are still highly genetically diverse (Alejandro Bonifacio, personal communication), due to the extensive use of landraces and lack of modern breeding practices in quinoa production. Secondly, the conditions under which the quinoa was grown may have been too ideal and too artificial for quinoa growth and all treatments grew equally well, regardless of *Bacillus* treatment. The conditions in which quinoa is grown in the Andean altiplano are incredibly harsh (intense solar radiation, nutrient poor soils, saline soils, drought and frost) and these conditions do not compare to the highly controlled conditions held within the growth chambers used in these trials.

The lack of plant growth promotion may have been related to a lack of effective colonization by the inoculated *Bacillus* species. Non-inoculated controls used in these experiments, excluding the rifampicin resistant trial, always had high levels of endospore forming bacteria. It is unknown where these competing bacteria, likely *Bacillus* species, were introduced into the experimental system.

These experiments highlight the need to introduce selectable markers into the bacteria being studied in order to reliably measure colonization. Attempts to develop a general selective *Bacillus* medium, such as Salt-V8 agar for *B. subtilis* (124), were unsuccessful, likely due to the diverse species used in this study and lack of selectable traits, such as unique nutritional requirements. The selection used in these studies, pasteurization to select for endospore formers, was effective, but could not eliminate other aerobic endospore formers that also colonized the

roots of the trial plants. Therefore, it was necessary to use rifampicin resistant mutants to determine colonization level. In the Rif^R studies, there were significantly different levels of colonization for all inoculated treatments compared to the uninoculated control. Future colonization studies, such as those performed in Bolivia, would be easier with the use of an antibiotic selection marker.

In order to identify *Bacillus* that can enhance the growth of quinoa, a rapid high throughput screening methodology must be developed. Realistically, the utility of this method would hinge on the ability to screen *Bacillus* isolates for desired qualities (ideal species, plant hormone production, ability to antagonize) and a rapid method to measure plant growth promotion and colonization *in planta*. The trials performed in this series of experiments did not demonstrate any enhancement of plant growth, nor were consistent colonization levels established. However, the use of antibiotic resistance as a method to screen a high number of very diverse species of *Bacillus* does seem to be a promising avenue for future research and screening by our Bolivian collaborators.

Chapter 3

Phosphate Solubilizing *Bacillus* Associated with *Chenopodium species*

Introduction

Phosphorus is essential for all life. After nitrogen, phosphorus is often the most limiting resource in agroecosytems (132) but, unlike nitrogen, phosphorus cannot be fixed from the atmosphere. Therefore, nearly all phosphorus in the soil comes from human inputs or the soil parent materials. One major human input is synthetic phosphate fertilizers, but as sources of these fertilizers become depleted, use of phosphate fertilizers becomes more expensive and less sustainable. Methods to utilize existing, but less available, soil phosphorus *in situ* are being studied to provide sustainable methods of phosphorus management. One such area of research is the use of plant associated phosphate solubilizing bacteria, which are capable of converting insoluble phosphates to plant available phosphates.

Soil Phosphorus Cycle

The soil phosphorus cycle is complex. Soil phosphorus exists in three differentially extractable pools: inorganic, organic and plant available orthophosphate pools (solution P) (134). The conversion of organic phosphorus to plant available orthophosphate is termed mineralization, whereas the reverse process is immobilization. The conversion of inorganic phosphorus to plant available orthophosphate is called solubilization or desorption and the reverse process is termed fixation or adsorption (134). Inputs to the soil phosphorus pools include fertilizers, manures and sludges and phosphorus is lost from soil through plant uptake and leaching (134).

Phosphorus exists in diverse forms in the soil (19). Inorganic phosphorus exists in several forms depending on the pH of the soil. Calcium phosphates (apatites) are more common in alkaline soils (pH > 7). Aluminum phosphates (variscite) and iron phosphates (strengite) are more common in acidic soils. Organic forms of phosphorus can be found in all soils and include inositol phosphates, phospholipids, nucleic acids, and biomass. The plant available orthophosphate pool is most available to plants around pH 7.0 and consists of monobasic and dibasic orthophosphates. Phosphorus availability in soils is affected by soil carbon (3) and soil nitrogen levels (30).

Phosphate Solubilizing Bacteria: Diversity and Modes of Action

One potential way to decrease the need for synthetic phosphorus fertilizers is through the use of phosphate solubilizing bacteria (PSB). PSB release plant available phosphates from both inorganic and organic soil phosphorus pools. The process by which PSB release phosphate is termed solubilization (a term which here covers both mineralization and solubilization). Phosphate release is accomplished through the production of organic acids, proton efflux, phosphatase and phytase production and the extracellular oxidation of glucose (104). The most commonly isolated and researched genera of PSB include *Pseudomonas*, *Bacillus*, and *Rhizobia* (69). Other bacterial genera have been shown to solubilize phosphate and these include *Burkholderia, Enterobacter, Escherichia, Pantoea, Erwinia* and *Agrobacterium* (69).

PSB have several different modes of phosphate solubilization based on the type of bacteria. These unique methods of phosphate solubilization can be used to track specific types of PSB in the environment. For example, direct oxidation of glucose is a reaction that only takes place in gram negative bacteria and is an important means of phosphate solubilization (58). In this reaction, glucose is oxidized to gluconic acid by the enzyme glucose dehydrogenase with its

cofactor pyrroloquinoline quinone (PQQ). PQQ can be used as a marker of the phosphate solubilizing phenotype in *Pseudomonas* (89). Similarly, phosphatase genes of *Bacillus* species can be used as markers of the phosphate solubilizing phenotype (68).

The production of organic acids is thought to be the most prevalent means of bacterial phosphate solubilization. The most commonly produced organic acids are citric, gluconic, lactic, succinic and propionic acids (24). The proposed reaction for solubilization of inorganic phosphorus by organic acids is as follows:

$$CaPO_4 + HA = HPO_4 + CaH \text{ (57)}$$

Phosphate Solubilizing Bacteria: Plant, Soil, and Microbial Interactions

PSB have many complicated interactions with plants, soil and other microbes. PSB can be beneficial to plants. PSB can promote plant growth by increasing nutrient availability, serving as biological controls agents, producing plant hormones and by enhancing other plant-microbe mutualisms, such as rhizobial and mycorrhizal interactions (51; 104). The phosphate solubilizing phenotype of PSB can be triggered by root exudates which suggests a mutualistic nutrient exchange relationship (42; 58).

PSB can also interact with other plant beneficial microbes. PSB can have synergistic or neutral effects when co-inoculated with rhizobia (106; 131). PSB are thought to provide phosphorus to supply the high energy requirements of the rhizobial nitrogen fixing reaction. If a rhizobium is able to solubilize phosphate itself, there will be no effect of co-inoculation with a PSB (106). PSB can also interact with mycorrhizal fungi to provide phosphorus to plants (122).

However, it is the interaction of PSB with soil physical and chemical properties that we understand the least. The level of soil phosphorus affects the diversity and abundance of PSB

(82). Phosphorus deficient soils have been shown to contain PSB with plant growth promoting phenotypes (41). Conversely, PSB populations were similar when sampled from rhizospheric samples and bulk soil of a rock phosphate mine and nearby non-mine soil (103) which indicates that phosphorus levels did not affect PSB populations in this case. Populations of bacteria involved in soil phosphorus cycling decrease with depth in soil (3), but most other bacteria follow a similar distribution.

The ability of PSB to solubilize phosphorus in acidic soils has not been properly described. The role of organic acid production in releasing phosphates from inorganic pools in acidic soils is unclear (115), but some suggest that a cation exchange occurs in order to release soluble phosphate (57).

The Role of Phosphorus in Plant Disease

Another benefit that PSB may have by providing phosphorus to plants is a concurrent reduction in disease, however, there is no good evidence for this claim. A substantial review of recent literature on the role of phosphorus in disease development in plants yielded conflicting results (96). For example, the authors reviewed several independent papers that examined the role of phosphorus in eight different diseases of wheat and found that increased phosphorus had either a positive or negative effect on disease depending on the pathogen (96). Similarly, when the effects of phosphorus on the same genus of pathogen (*Fusarium oxysporum*, for example) were examined in different crops, the authors found that disease would either increase or decrease depending on the crop (96). Therefore, it is unlikely that phosphorus would play a significant role in effectively reducing diseases in quinoa.

Clearly, there is a great deal of research that still needs to be performed to determine the ecological roles of PSB. In these studies, we examined populations of *Bacillus* isolated from

Chenopodium species to determine if specific species of *Bacillus* were capable of tricalcium phosphate solubilization. This population based approach was used to reveal patterns of the tricalcium phosphate solubilizing phenotype within the genus *Bacillus*.

Materials and Methods

Isolation of *Bacillus* from *Chenopodium species*

Three populations of *Bacillus* from *Chenopodium species* were obtained in this study. The first *Bacillus* population was isolated from *Chenopodium album* collected from the Russell E. Larsen Research Farm at Rock Springs during September 2010. The second *Bacillus* population was isolated from *Chenopodium quinoa* collected in Ecuador during July 2011. The final *Bacillus* population was isolated from *C. quinoa* collected in Tiraque Province, Bolivia during February 2012. *Bacillus* isolates were isolated from *C. album* samples immediately after sampling in the field, whereas isolation of *Bacillus* from *C. quinoa* began as soon as samples arrived from South America, which was approximately one and a half weeks after initial collection. Plant material arrived in good condition and there were few visible signs of tissue deterioration. A pasteurization step ensured that only aerobic endospore forming bacteria were isolated, even if secondary contaminants had developed.

Bacillus was sampled from *C. album* roots and leaves and from *C. quinoa* roots, shoots and leaves. For isolation from roots, all excess soil was brushed from the root and the root was rinsed with distilled water. The root was stirred in 10% commercial bleach with Tween 20 for 5 minutes. The root was removed from the bleach, blotted on sterile paper towels, rinsed twice in sterile distilled water, blotted dry on sterile paper towels and placed in a sterile sample bag.

Roots were mechanically disrupted with a hammer and 5 mL of sterile 0.1 M phosphate buffer was added to the sample bag.

For foliar and stem isolation of *Bacillus*, 5-7 quinoa leaves or a 2-3 inch portion of stem were placed into a sterile sample bag and washed in 25 mL of sterile water by mixing in the stomacher blender (Bagmixer 100, Intersciences, Saint Nom, France) at its lowest setting for one minute. The water was discarded and the process repeated twice. The leaves or stem portion were macerated with a hammer and 5 mL of 0.1 M phosphate buffer was added to the sample bag.

All samples were homogenized after smashing in the stomacher blender for 1 minute at maximum speed. Liquid was drawn off and placed in sterile 1.5 mL tubes. Samples were heated at 75° C for 15 minutes to kill vegetative cells. One hundred µL of sample was plated onto TSA in triplicate and incubated for 24-48 hours at 30° C. At this time, 3-5 unique morphologies were chosen from each plate and streaked onto TSA in pure culture. At this time, isolates were assigned their permanent designation (for Pennsylvania population: CA#, for Ecuador population: EC#, for Bolivia population: BV#).

Storage of Isolates

Isolates were streaked onto 60 mm petri plates with either TSA (Ecuadorean, Pennsylvanian) or YDC agar (Bolivian) and stored at 4° C. Additionally, isolates were grown overnight in TSB and transferred to glycerol (20% final concentration of glycerol) and stored at -20° C. For Ecuadorean and Bolivian isolates, a toothpick was touched to a freshly isolated colony which was transferred to 50 µL of sterile milliQ water and boiled in 1.5 mL microfuge

tubes for 10 minutes. Tubes containing the boiled suspension were stored at -20° C and the suspensions were used as DNA templates for PCR.

PCR and Sequencing of *Bacillus* isolates from *Chenopodium* species

Isolates were identified to species through sequencing of direct colony PCR products. Direct colony PCR methods were adapted from Woodman (2008, (137)) and Melnick et al. (2011, (87)) to amplify an approximately 850 bp portion of the 16S ribosomal RNA. Pure cultures were streaked onto TSA plates and incubated at 30° C for 24-48 hours. At this time, a colony or portion of a colony was picked with a toothpick (so that bacteria could just be seen on the tip) and placed into 20 µL PCR reaction mix. Similarly, 1 µL of bacterial DNA from boiled suspensions could be used as the reaction template. The PCR reaction mix for this study consisted of 10 µL of Gotaq Green master mix (Promega Corporation, Madison, WI), 1 µL of 30µM 530f (5' to 3' GTGCCAGCMGCCGCGG), 1 µL of 1392R (5' to 3' ACGGGCGGTGTGTRC), and 8 µL of sterile, nuclease-free water. The PCR conditions were as follows: 5 minutes at 94° C, followed by 25 cycles of 30 seconds of 94° C, 30 seconds of 55° C, 60 seconds of 72° C, and finally followed by a final extension at 72° C for 5 minutes. PCR products were purified with ExoSap IT (Affymetrix/USB, Santa Clara).

Sequencing was done at the Pennsylvania State University Genomics Core Facility using an Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, Foster City, California). Isolates were identified to species or species group using BLAST (NCBI) or the Ribosomal Database Project.

Qualitative Screen for Tricalcium Phosphate Solubilization

NBRIP media (92), which contains tricalcium phosphate, was used for qualitative screening of *Bacillus* isolates for phosphate solubilization. Because previous studies have indicated that some isolates of bacteria seemed to "lose" their ability to solubilize phosphate with repeated culturing, a 5 week-long experiment was designed to screen bacteria for their ability to solubilize phosphate under conditions of repeated sub-culturing. A 100 mm petri dish was divided into quadrants in order to test four isolates on one plate. Isolates were stabbed in triplicate into NBRIP media, and plates were incubated at 30° C. Each isolate was transferred from the previous week's stab inoculation to a fresh plate after 1 week. This process was repeated for 5 weeks, so there were 5 total passages of each *Bacillus* isolate in the media. Plates were retained for two weeks and were evaluated for cleared halos, produced by organic acids or phosphatases, around the stab inoculation for the first and second week of growth. A halo, which indicated tricalcium phosphate solubilization was occurring, was rated as a solubilization value of 1 and the absence of a halo was rated as a solubilization value of 0. After five sequential passages (with evaluations during the first and second week of each passage), the solubilization values for each isolate were totaled. If a halo was present in more than 50% of evaluations (a rating greater than 5), that isolate was qualitatively phosphate solubilizing.

Phylogenetic Analysis

Phylogenetic analyses of bacterial 16S sequences were performed using MEGA5 (Tamura, et al. 2011). Sequences were examined and cleaned using ChromasLite 2.01 (Technelysium Pty Ltd). Sequences were aligned using MUSCLE (Edgar, 2004) and manually adjusted. Maximum likelihood trees were created from aligned sequences using a Kimura 2 parameter model with a gamma distribution with invariant sites. Trees were tested using the

bootstrap method with 100 replications. Complete phylogenetic trees were created with all sequences from the separated populations, and smaller trees were constructed using sequences of *B. cereus, B. simplex, B. megaterium*, and *B. subtilis* groups. On each tree, the qualitative phosphate solubilizing ability of each isolate was mapped by hand using a color coded system (Figure 3-1).

Growth Assays with Phosphate Solubilizing *Bacillus*

In two trials, the effects on quinoa growth of a phosphate solubilizing strain of *Bacillus megaterium* (BV109) were compared to a non-phosphate solubilizing strain of *Bacillus simplex* (BV110). Both isolates were obtained from the same quinoa plant. The isolates were inoculated onto quinoa seeds and quinoa growth was evaluated at the end of the experiment. Both experiments were carried out in a Conviron growth chamber with day temperatures of 24° C and night temperatures of 21-22° C with 65% relative humidity. Lights were set to a 12 hour day/night cycle. Inoculum was produced as described in the previous chapter.

Conetainer Assay

In this assay, the ability of BV109 and BV110 to promote plant growth in the presence of four phosphorus sources was tested. The four phosphorus sources were tricalcium phosphate, iron phosphate, aluminum phosphate (all Sigma-Aldrich, St. Louis, MO) and rock phosphate (Espoma, Millville, NJ). For each phosphorus source, the amount added was adjusted so that 250 mg of elemental phosphorus was added to each 500 mL (approximately 500 g) of potting medium. The potting medium used in this study consisted of 2 parts washed playground sand, 1 part perlite and 1 part vermiculite. 500 mL of potting medium was added to a paper bag, the phosphorus source was added, the media was autoclaved for 1 hour, dried overnight and

autoclaved an additional hour 24 hours after the first autoclaving. The autoclaved potting medium was added to bleached Conetainers (500 mL capacity, diameter, 6.5 cm, 25 cm deep) and the media was watered. Quinoa 'Faro' seeds were placed 5 mm deep in each Conetainer and the quinoa seeds were inoculated with 1 mL of *Bacillus* inoculum (log CFU/ml for BV109 was 8.0 and for BV110 was 7.9) and covered with planting medium.

Plants were fertilized with 10 mL of full strength Hoagland's without phosphorus solution every other day of the experiment. This experiment ran for 39 days. At the end of the experiment, plants were harvested and fresh and dry above ground biomass, shoot height, and dry root biomass were recorded. All fully expanded leaves with petioles were harvested, dried overnight at 50° C and submitted for nutrient analysis at the Penn State Agricultural Analytical Lab.

Test Tube Assay

In this trial, plants were grown in 50 mL test tubes containing Soilmoist, (JRM Chemical, a polyacrylamide gel used in horticultural applications), prepared by mixing 30 mL of Soil Moist in 1L of full strength Hoagland's solution without phosphorus, amended with 5 g of tricalcium phosphate and 0.03 g bromophenol blue, as a pH indicator. One half mL of *Bacillus* inoculum (log CFU/mL for BV109 was 8.0 and for BV110 was 7.9) was added to the Soilmoist after planting quinoa 'Faro' seeds in the tubes. Six replicates of each treatment (BV109, BV110, no bacteria) were included and tubes were randomized in a test tube rack for this experiment.

The duration of this experiment was 28 days. After harvesting, data on fresh and dry above ground biomass, shoot length, root length and dry root biomass were collected.

Results

<u>Tricalcium Phosphate Solubilizing *Bacillus* species</u>

Three *Bacillus* populations were used in these studies. The first population (CA), from Pennsylvanian *Chenopodium album*, consisted of 194 isolates, of which 177 were sequenced and 175 were screened for phosphate solubilization. The Ecuadorean (EC) population from *C. quinoa* consisted of 335 isolates of which 327 were screened for phosphate solubilization and 302 sequences were obtained. The Bolivian population (BV), obtained from *C. quinoa*, consisted of 342 isolates; all isolates were screened for phosphate solubilization, and 330 isolates were sequenced. The percentages of isolates in each population that solubilized tricalcium phosphate were similar (CA: 57% (99 of 175), EC: 62% (204 of 327), BV: 69% (237 of 342)).

Within the three populations, there was variability among isolates in tricalcium phosphate solubilizing ability depending on what portion of the plant the isolate was obtained from (Table 3-1). The percentage of tricalcium phosphate solubilization in root obtained isolates was similar for the Pennsylvanian and Ecuadorean populations, but was higher in the Bolivian population. The percentage of foliar isolates capable of tricalcium phosphate solubilization in each population was higher than the percentage of root isolates capable of tricalcium phosphate solubilization. The percentage of tricalcium phosphate solubilizing stem isolates compared to root isolates was higher in the Ecuadorean population, but lower in the Bolivian population.

Table 3-1 Percentage of *Bacillus* populations capable of phosphate solubilization in vitro by total population and by section of plant that isolates were obtained

Population	Total % P-solubilizing	% Root P-solubilizing	% Stem P-solubilizing	% Leaf P-solubilizing
Pennsylvania	57%	54%	n/a	67%
Ecuador	62%	58%	73%	66%
Bolivia	69%	79%	56%	88%

The percentage of tricalcium phosphate solubilizing isolates varied by species (or species group) of *Bacillus*. Four *Bacillus* species groups were common to each population (*B. cereus, B. simplex, B. megaterium*, and *B. subtilis*). *B. subtilis* and *B. megaterium* species groups were consistently found to be capable of solubilizing tricalcium phosphate (with percentages at or near 100% for each population, Table 3-2). *B. simplex* and *B. cereus* species groups were variable in their ability to solubilize phosphate. The *B. simplex* group had a range of 28-73% of all isolates capable of solubilizing phosphate, while the *B. cereus* group had 11-66% of all isolates with the same capability. For these four species groups, the patterns of phosphate solubilization are seen when mapped onto a phylogenetic tree of the four species groups (Figure 3-1)

There were many other species of *Bacillus* identified in each of the three populations, but these species often only had 1-5 isolates, which was not enough to draw conclusions. *Paenibacillus* species were also common to the three populations, but these are a different genus of aerobic endospore forming bacteria. Two species, *B. gibsonii* and *B. drentensis*, were common to two populations. For *B. gibsonii*, no isolates were capable of solubilizing phosphate in either the Bolivian or Ecuadorean population, whereas for *B. drentensis* 75-100% of isolates could solubilize phosphate in the Pennsylvania and Bolivia population.

Table 3-2 Number and percentage of isolates, by species group, from three populations capable of solubilizing tricalcium phosphate in-vitro. For each species group, the number of isolates capable of solubilizing phosphate out of the total isolates of that species group was used to determine the percentage of each species group capable of phosphate solubilization.

***Bacillus* species group**	Pennsylvania Population	Bolivia Population	Ecuador Population
B. subtilis group	20 of 20 (100%)	91 of 91 (100%)	44 of 46 (96%)
B. cereus group	13 of 44 (30%)	2 of 19 (11%)	22 of 33 (66%)
B. simplex group	7 of 25 (28%)	48 of 66 (73%)	58 of 130 (45%)
B. megaterium group	47 of 47 (100%)	20 of 20 (100%)	4 of 4 (100%)
Paenibacillus group	2 of 2 (100%)	56 of 66 (85%)	47 of 52 (90%)
B. gibsonii	n/a	0 of 51 (0%)	0 of 2 (0%)
B. drentensis	3 of 4 (75%)	n/a	12 of 12 (100%)

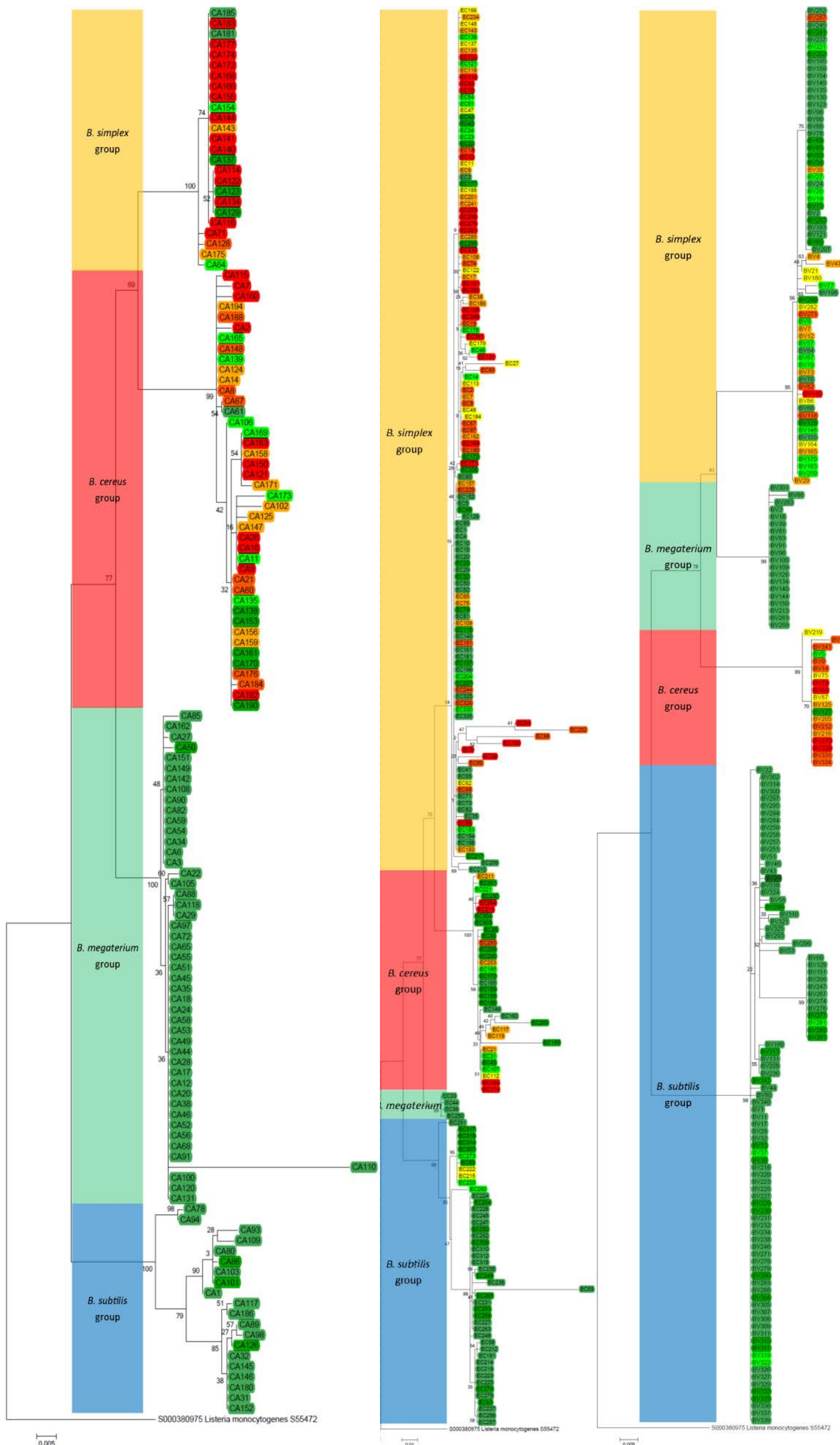


Figure 3-1: Phylogenetic trees showing the patterns of tricalcium phosphate solubilization in populations of *Bacillus* from *Chenopodium album* from Pennsylvania (CA), *Chenopodium quinoa* from Ecuador (EC), and *C. quinoa* from Bolivia (BV). Tricalcium phosphate solubilization was rated on a scale of 1-10: 0=■, 1-2=■, 3-4=■, 5=■, 6-7=■, 8-9=■, 10=■

Growth Assays with Phosphate Solubilizing Bacteria

Conetainer Assay (Figure 3-3)

To analyze the data collected in this experiment, the general linear model was used in Minitab 16 (Minitab, State College, PA) with fresh and dry shoot biomass, shoot height and dry root biomass as the responses and the model contained the bacterial treatment, the phosphorus treatment, the interaction between the two treatments and the blocking used in this experiment. Blocking (p-value ranged from 0.197-0.962) and the bacteria and phosphorus source interaction (p-value ranged from 0.27-0.906) were not significant for any of the responses. Shoot height was significantly different based on bacteria treatment (p=0.03) and phosphorus source (p=0.017). Based on Tukey's HSD, BV109 had a significantly lower mean shoot length than no bacteria or BV110, while the use of tricalcium phosphate led to a significantly lower shoot length than iron, aluminum or rock phosphates. Phosphorus source significantly affected dry root biomass (p=0.012) with rock phosphate having significantly lower dry root biomass compared to iron, aluminum and calcium phosphates, based on Tukey's HSD.

For the nutrient analysis in regards to phosphorus, no significant differences (p=0.192) were found for any of the bacterial treatments within each phosphate source. The phosphorus levels of plants with the tricalcium phosphate treatment (Figure 3-2) were significantly higher (p=0.00) than the other three sources and phosphorus levels were also significantly higher for aluminum phosphate compared to rock phosphate, based on Tukey's HSD. Tricalcium phosphate is the most soluble of the four calcium sources used, so it is reasonable that phosphorus levels would be higher in plants with a tricalcium phosphate treatment.

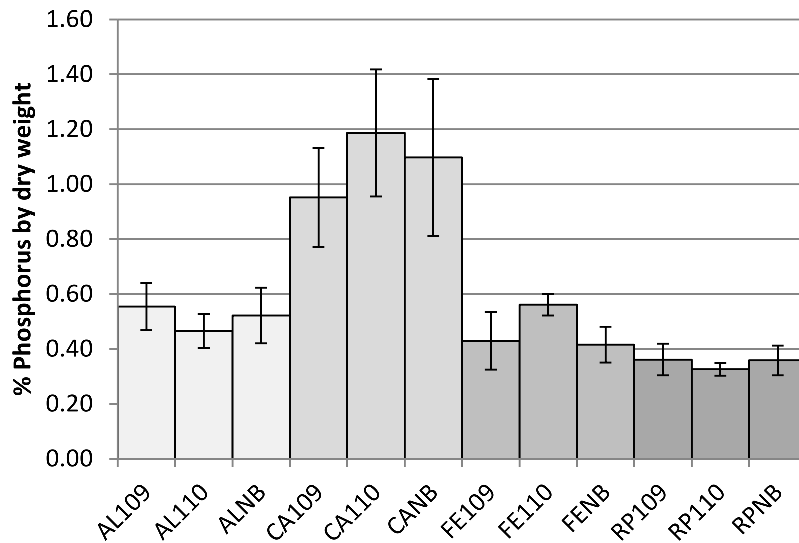


Figure 3-2 Percent phosphorus on a dry weight basis for Conetainer assay comparing effects of a phosphate solubilizing *B. megaterium* BV109 to a non-solubilizing *B. simplex* BV110 on quinoa growth with four phosphate sources: aluminum phosphate (AL), tricalcium phosphate (CA), iron phosphate (FE) and rock phosphate. The controls (NB) were not inoculated with bacteria. Error bars are standard deviation.

Test Tube Assay

One-way ANOVA was used to analyze the results of this experiment, differences in treatment means were not significant (Figure 3-4) for root length (p=0.608), fresh shoot biomass (p=0.491), dry shoot biomass (p=0.796), dry root biomass (p=0.937), or shoot length (p=0.125).

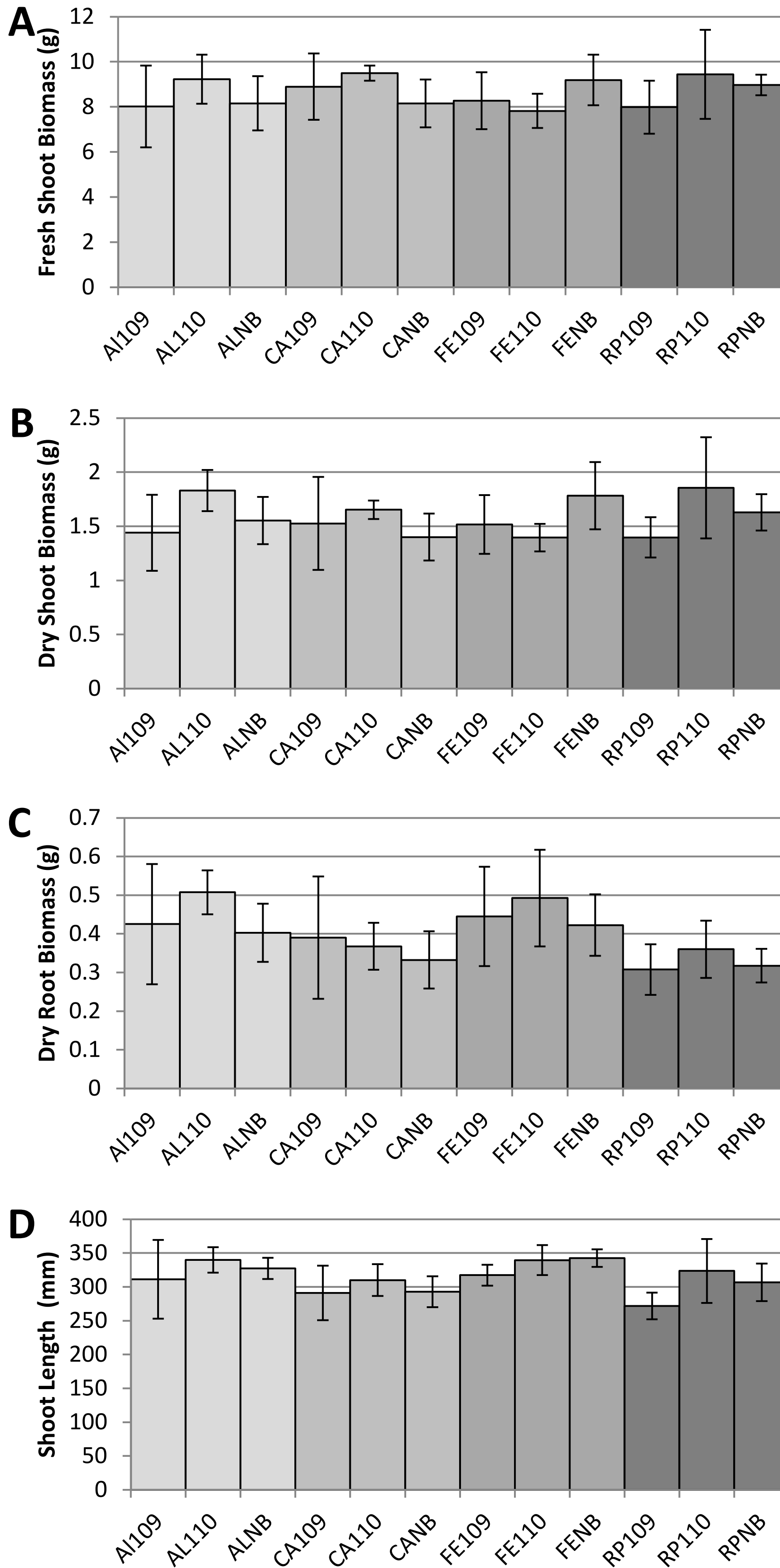


Figure 3-3 Results of phosphate solubilizing *Bacillus* trial in which a non-solubilizing *Bacillus simplex* (BV110) was compared to a phosphate solubilizing *Bacillus megaterium* (BV109). Plant growth was compared using four different phosphate sources: aluminum phosphate (AL), tricalcium phosphate (CA), iron phosphate (FE) and rock phosphate (RP) and a non-bacteria (NB) inoculated control was included. Fresh aboveground biomass (A), dry aboveground biomass (B), dry root biomass (C) and shoot length were compared for all treatment combinations.

Error bars are standard deviation.

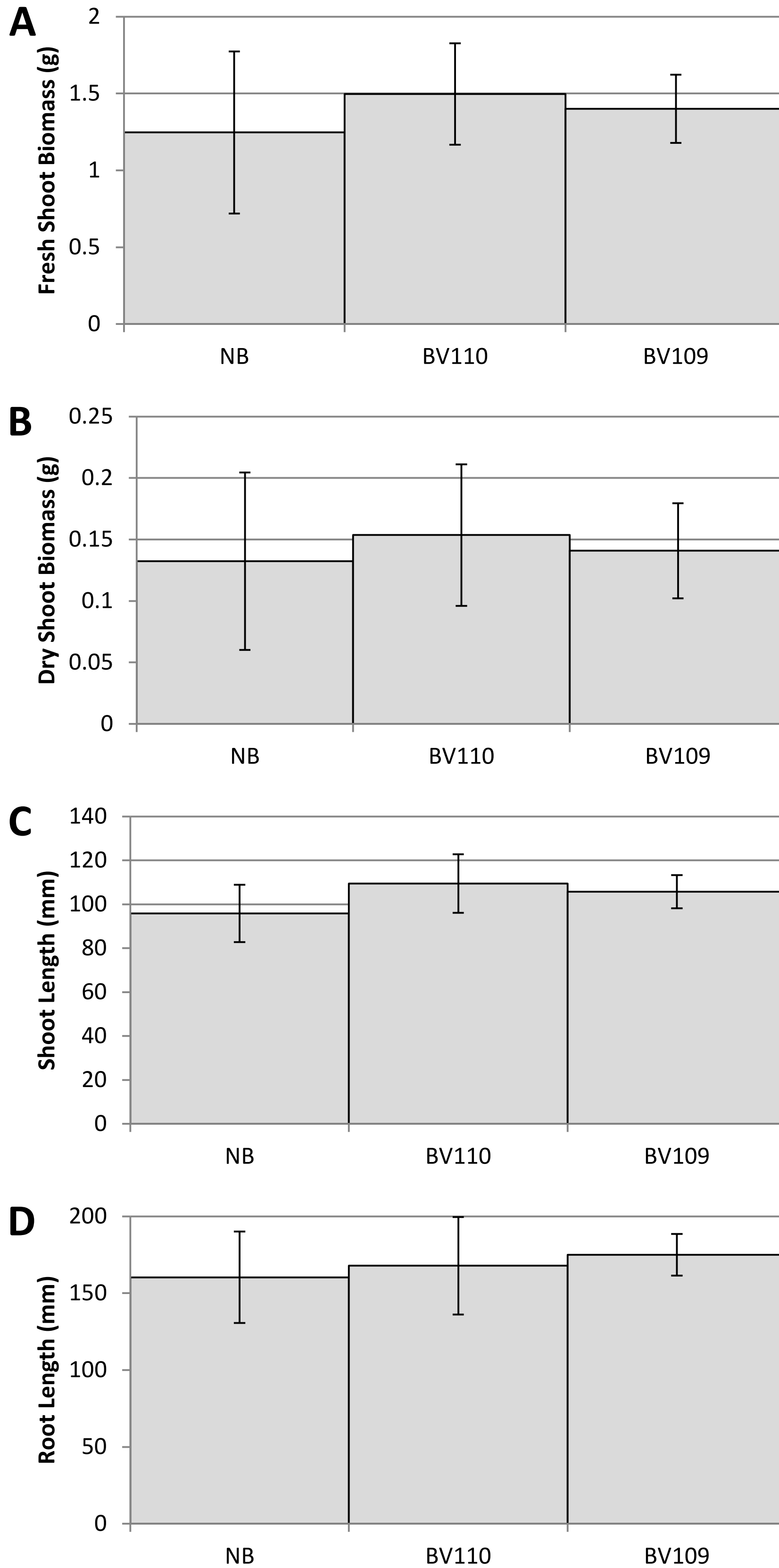


Figure 3-4 Results of phosphate solubilizing *Bacillus* trial in which a non-solubilizing *Bacillus simplex* (BV110) was compared to a phosphate solubilizing *Bacillus megaterium* (BV109). Plants were grown in Soilmoist amended with tricalcium phosphate and a non-bacteria inoculated control was included (NB). Fresh aboveground biomass (A), dry aboveground biomass (B), shoot length (C) and root length (D) were compared for all treatments. Significant differences were not observed for any treatment.

Error bars are standard deviation.

Discussion

A population based approach was used in these studies to determine if there was variability in the tricalcium phosphate solubilizing phenotype within the bacterial genus *Bacillus*. Three *Bacillus* populations, all from *Chenopodium* species, were chosen for this study to allow for comparison of the results. A population based approach was undertaken because the majority of PSB studies use the following pattern (41; 104): isolate random bacteria from substrate, screen for solubilization, choose best in-vitro solubilizers, and test on plants. These studies are beneficial because they allow for the study of specific isolates of bacteria and their roles in plant growth promotion, but these studies do little to illustrate the ecological role of the tricalcium phosphate solubilizing phenotype. If the tricalcium phosphate solubilizing phenotype truly is beneficial to plants, one could make the following assumptions: 1) PSB would be root associated and 2) the tricalcium phosphate solubilizing phenotype would be commonly found in specific groups of plant growth promoting bacteria. The incidence of PSB in the rhizosphere is higher than in bulk soil (104), which offers support for the first assumption. To answer the second assumption, one must examine a population of bacteria from different hosts to determine the distribution of the tricalcium phosphate solubilizing phenotype across bacterial species.

Three *Bacillus* populations were screened to determine the distribution of the tricalcium phosphate solubilizing phenotype within the genus. For all isolates screened, the Bolivian population had a slightly higher percentage (69%) of phosphate solubilizers, which may be due to the soil conditions from which the host quinoa plants were grown. Previous research (82) demonstrated that grassland soils with lower phosphorus content had higher incidences of phosphate solubilizing Pseudomonads. However, this grassland study did not examine differences in *Bacillus* populations directly. We have no baseline information on the Ecuadorean

and Bolivian soils in our study, so we cannot compare our results to the grassland study. Therefore, it would be beneficial to explore how soil phosphorus levels affect phosphate solubilizing *Bacilli*.

The species groups chosen in these studies were common to all three populations obtained in these studies. Historically, these groups were described because their members have similar phenotypes (50) or highly similar 16S sequences (78; 84), but our studies still indicate variability in the tricalcium phosphate solubilizing phenotype. The *B. cereus* species group contains *B. anthracis, B. thuringiensis*, *B. mycoides* and *B. cereus*. The major species included in the *B. subtilis* species group include *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus and B. licheniformis*. Although *B. simplex* has been lumped with the *B. megaterium* group, these two groups formed separate lineages based on 16S sequencing, so two separate species groups were used. Further resolution of both *B. megaterium* and *B. simplex* are needed to better resolve their taxonomic status in *Bacillus*.

In these studies, we sought to determine if tricalcium phosphate solubilization was limited to certain species of *Bacillus* or if tricalcium phosphate solubilization was a trait common to the genus. The results of the qualitative tricalcium phosphate solubilization assay paired with phylogenetic analysis indicate that the *B. megaterium* and *B. subtilis* groups are nearly universally capable of phosphate solubilization, whereas members of the *B. cereus* and *B. simplex* groups vary in their ability to solubilize tricalcium phosphate. Members of the *B. subtilis* group are commonly studied as plant growth promoting bacteria (71), which may indicate that the tricalcium phosphate solubilizing phenotype is tied to plant growth promotion.

Further studies are needed to determine why there is qualitative variability in the tricalcium phosphate solubilizing phenotype of *Bacillus* species. Our approach is not quantitative and a quantitative approach to determine the rates and amounts of phosphate solubilization by species would be useful for future studies.

There is some evidence gained in these studies which throws the ecological role of the tricalcium phosphate solubilizing phenotype into question. One would assume most PSB are root associated, but in this study we found higher percentages of phosphate solubilization in foliar isolates in all three populations and a higher percentage in stem isolates of the Ecuadorean population compared to percentage of phosphate solubilization of root isolates. The stem and foliar isolates could have originated from the soil and may require the phosphate solubilizing phenotype for portions of the lifecycle not associated with plants. Another possibility is that bacteria that survive in or on the stem or phyllosphere are root associated at some point in their lifecycles, but further studies are required to determine this. The diversity of foliar isolates may also be skewed to species of *Bacillus* that solubilize phosphate. In this study, there were many *B. subtilis* isolated from the leaves, all of which were phosphate solubilizing. The ecological role of the phosphate solubilizing phenotype in other plant parts is unknown, but would be interesting to pursue in further studies with other plant species.

If the tricalcium phosphate solubilizing phenotype is truly important for plant growth promotion, one would expect to see differences in plants inoculated with either a phosphate solubilizing or a non-phosphate solubilizing isolate grown under phosphate limited conditions. Although differences were found between isolates in the qualitative ability to solubilize phosphate, no differences in plant growth were observed when plants were inoculated with either a phosphate solubilizing or non-phosphate solubilizing strain. There may be several reasons why

no differences were observed. First, the genetic variability of quinoa ‘Faro’ may be higher that what is needed to obtain reliable data. Phosphorus levels used in the potting media or soil moist for the experiments may have been too high or the plants were capable of obtaining sufficient phosphorus levels using their own root adaptations. The non-solubilizing *B. simplex* was capable of producing IAA based on screens performed with a Bolivian collaborator (Claros, PROINPA). This phenotype may have accounted for equivalent growth between the phosphate solubilizing and non-solubilizing isolates.

In conclusion, the results of these studies indicate that the tricalcium phosphate solubilizing phenotype is more common in the *B. subtilis* and *B. megaterium* groups compared to the *B. cereus* and *B. simplex* groups, at least when associated with *Chenopodium* species. In plant based studies, no significant differences in plant growth were observed between plants inoculated with either a phosphate solubilizing or non-solubilizing isolate of *Bacillus*, but no differences were observed when comparing the growth of the inoculated plants to uninoculated controls. These studies provide some insights into the distribution of the tricalcium phosphate solubilizing phenotype in one genus of bacteria, but the overall ecological role of PSB is still unclear and deserves more research.

Chapter 4

Quinoa Downy Mildew: Phylogeny of *Peronospora variabilis* and Detection of *P. variabilis* in Quinoa Seeds and Plant Tissue

Introduction

For all its promise, quinoa (*Chenopodium quinoa*) is limited by one key disease, quinoa downy mildew. Quinoa downy mildew is caused by the obligate pathogenic oomycete, *Peronospora variabilis* (26) (formerly *Peronospora farinosa* f.sp. *chenopodii*). In Peru, studies on yield loss due to *P. variabilis* found that losses could range from 6-99% (36), but under disease conducive conditions, the range narrowed to 33-99% yield loss. Yield loss levels in other countries have not been documented, even though quinoa downy mildew has been reported in Canada (120), Denmark (38), the United States (119) and India (76).

Quinoa downy mildew has been reported across the world. The likely source of inoculum for quinoa downy mildew is from seedborne oospores, (35) as these seedborne oospores can initiate downy mildew infections in emerging quinoa seedlings. However, there are several reports of downy mildew being observed on *Chenopodium* weed species near infected quinoa fields (38; 120). These reports did not examine if *Peronospora* species on these weeds were infective to quinoa or vice versa, but it is possible that *Peronospora* pathogens of *Chenopodium* weed species could also infect quinoa. In Bolivia and Ecuador, weedy *Chenopodium* species are thought to serve as reservoirs of *P. variabilis* between quinoa growing seasons (G. Plata and J. Ochoa, *unpublished*), though oospores are likely another source of overseasoning inoculum.

The importance of seedborne oospores in the quinoa downy mildew disease cycle is uncertain. *P. variabilis* is heterothallic (34) and a plant would need to be infected with both mating types for oospore formation to occur. Oospores do allow for over-seasoning of the pathogen (35) and oospores are also reported in leaf tissue, which becomes incorporated into the soil. However, it is unclear if initial inoculum for quinoa infections at the beginning of the growing season comes from seedborne oospores, soilborne oospores or sporangia blown in from infected weedy *Chenopodium* species.

Seedborne oospores could account for the global spread of quinoa downy mildew. However, a systematic visual screening of quinoa seed coats found that only 19 of 128 Peruvian quinoa seed lots contained oospores (35). These tests, however, only screened 100 seeds of each seed lot and the number of oospores detected ranged from 1-8 (maximum of 20, for one lot). These visual detection methods are not sensitive or rapid enough to detect the pathogen effectively. Molecular methods for rapid seed detection of *P. variabilis* would allow for screening of larger samples of quinoa seed lots and would serve as a first step in quinoa seed certification. Clean quinoa seed could be an important component of integrated management options for quinoa downy mildew.

Management strategies for quinoa downy mildew are lacking. Downy mildew resistant quinoa lines are being developed (Alejandro Bonifacio and Amalia Vargas, *unpublished*). Fungicides (39) are used in quinoa production but consumer demand is for organic quinoa. Therefore, there is a need for researching the use of biological control or biorational pesticides for the control of quinoa downy mildew. No documented studies have been found pertaining to biological control of quinoa downy mildew.

This research had three objectives to aid in sustainable management of quinoa downy mildew: 1) to develop a rapid detection method for the quinoa downy mildew pathogen, 2) to determine if weedy *Chenopodium* species harbor the pathogen, and 3) to determine if biological control of quinoa downy mildew is feasible.

Materials and Methods

Discovery of Quinoa Downy Mildew in Pennsylvania (119)

Quinoa plots were established at the Russell E. Larsen Research Farm at Rock Springs (Centre County) and at the Southeastern Research and Extension Center (SEAREC, Lancaster County, PA). Twelve lots of seed (1 bred variety, 11 lines, all likely land races, purchased from grocery stores) were planted in late May in a randomized complete block design with three replicates at each of two Pennsylvania locations. These plots were primarily established to monitor for diseases. In late July, symptoms similar to quinoa downy mildew were observed on quinoa plants at both locations. No downy mildew like symptoms were noted on any *Chenopodium* weeds in either location. Samples were collected for further analysis. Based on results of sequencing (29) and morphology (26), the causal agent was *Peronospora variabilis*. Koch's postulates were validated in greenhouse and growth chamber studies (see Appendix 1 for full report).

Maintenance and Storage of *Peronospora variabilis* Lines

Two lines of *P. variabilis*, one from the research farm at Rock Springs and one from SEAREC, were maintained in detached quinoa leaves (35). Sporangia were vortexed from 3-5 sporulating leaves in two mL of sterile milliQ water. The sporangia suspension was poured and spread on 1% water agar in a 100 mm petri dish. Young quinoa leaves were placed on the agar

so that the top of the leaf was touching the sporangia suspension and agar. Petri dishes were sealed with Parafilm and placed in a brown paper bag to exclude light for 24 hours. The plates were maintained at 20° C with a 16 hour day, 8 hour night cycle. Lines of *P. variabilis* were cycled every 4-7 days, depending on the rate of sporangia development.

Rapid, reliable methods for storing sporangia of *P. variabilis* were also tested, because it is time consuming and not always possible to cycle the pathogen every 4-7 days. Three methods were tested: storage at -20° C of sporulating leaves on water agar (35), storage of sporulating leaves at -20° C on moist filter paper (35), and storage in liquid nitrogen (20). The simplest storage method was to prepare an extra plate during maintenance of *P. variabilis* lines, wait until heavy sporulation occurred, seal the plate with 3 layers of Parafilm to prevent desiccation, place in the refrigerator for a short period to cool, and then place the plate at -20° C. Similarly, heavily sporulating leaves could be rolled in strips of moistened filter paper, placed in a 2 mL microfuge tube and stored at -20° C. For storage in liquid nitrogen, sporulating leaves were shaken in 15% DMSO and aliquoted into 2 mL cryo-vials. Another method to preserve sporangia in liquid nitrogen was to shake sporangia from leaves, transfer to microfuge tubes, spin at 13.3K rpm for 3 minutes, decant any liquid and resuspend the sporangia in 15% DMSO, and transfer the suspended sporangia to cryovials. The vials were first frozen overnight at -20°C and then were transferred to liquid nitrogen.

The viability of sporangia from these methods was tested by removing sporangia from their storage and testing infectivity in detached leaves (the same methods used to maintain *P. variabilis* lines). Isolates remained viable for at least six months.

Test of *Peronospora variabilis* Mating Types

To determine if the Landisville and Rock Springs lines of *P. variabilis* were capable of mating and producing oospores, sporangia of both lines were mixed. The mixed sporangia were used to inoculate detached leaves in the method described previously. The leaves were incubated for one week and diseased leaves were microscopically examined for the presence or absence of oospores.

Development of *Peronospora variabilis* Specific Primers

Peronospora variabilis sequences (methods described in following section) were aligned using MUSCLE (Edgar, 2004) in MEGA5. The sequences were put into NCBI's Primer BLAST tool which generates primers and queries them against all GenBank accessions. Eight primer sets were ordered and tested for amplification of *Peronospora variabilis*. PCR conditions were optimized and primers were tested to determine if they also amplified *Peronospora farinosa* f.sp. *spinaciae* on spinach, *Bremia lactucae* on lettuce, *Pythium aphanidermatum* (pure culture), *Phytophthora infestans* on tomato, or *Plasmopara obducens* on impatiens.

Molecular Detection of *Peronospora variabilis* in Quinoa Seeds and Plant Tissue

Detection in Quinoa Seeds

The oospores of *P. variabilis* can be seedborne and current detection methods rely on germinating seeds under disease conducive conditions (35) or examining seeds microscopically (35). If oospores are detectable by these two methods, molecular detection of *P. variabilis* in quinoa seeds should also be possible. To address this objective, methods previously developed for microscopically examining seed washes of quinoa seeds for oospores (35) were adapted to

screen seeds molecularly. The seed wash method was utilized to reduce the numerous inhibitory compounds in seeds, such as phenolic compounds, that could interfere with DNA amplification.

Five grams (avg. 1,300 seeds) of quinoa seeds were placed in an autoclaved 250 mL Erlenmeyer flask with 50 mL of sterile milliQ water. Seeds were stirred for 30 minutes using a magnetic stir-plate at maximum speed. The contents of the flask were filtered through 4 layers of sterile cheesecloth into a sterile 50 mL centrifuge tube. The seed washes were centrifuged for 5 minutes at 4000 rpm. The supernatant from the seed washes was decanted and the pellet was suspended in the small amount of remaining liquid. The suspended pellet was transferred to a tube of Lysing Matrix A (MP Biomedicals, Solon, Ohio) and lysed in a FastPrep-24 Instrument (MP Biomedicals, Solon, Ohio) for two cycles of 45 seconds at speed 4.5. The suspension in which cells were lysed was the AP1 buffer and RNaseA from a DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) which were added before sample disruption and homogenization. The instructions of the Qiagen Plant Mini Kit were followed for the remainder of the DNA extraction. Obtained DNA was stored at -20° C for further analysis.

Two PCR based methods were used to analyze the DNA obtained from seed washes for the presence of *P. variabilis*. The first method used a semi-nested PCR protocol adapted from Cooke (2000 (29)) followed by sequencing of the PCR products. The second method used one set of *P. variabilis* specific primers (PV6, sequences in results section), followed by gel electrophoresis of PCR products and ethidium bromide staining to determine if *P. variabilis* was present in the seed washes.

The reaction mixture for the semi-nested PCR consisted of 12.5 µL of 2X GoTaq green master mix (Promega Corporation, Madison, WI), 1 µL of 5µM of each primer, 5.5 µL nuclease

free water and 5 µL of each template. The primer set used for the first reaction was DC6/ITS4 (29) and the primer set used for the second PCR was ITS6/ITS4 (29). The conditions for the semi-nested PCR are as followed: an initial denaturation step of 95° C for 2 minutes, followed by 30 cycles with denaturation for 20 seconds at 95° C, annealing at 55° C for 20 seconds, elongation at 72° C for 50 seconds, and the cycle concluded with a final elongation step of 72° C for 10 minutes (29). PCR products were cleaned with ExoSap IT (Affymetrix/USB, Santa Clara, California) and submitted for sequencing at the Penn State Genomics Core Facility. Products were sequenced using an Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, Foster City, California).

For identification of *P. variabilis* infected seeds using specific primers, the reaction mixture consisted of 10 µL of 2X Gotaq green master mix, 1 µL of 5µM of each primer, 7 µL of nuclease free water, and 1 µL of template DNA. The reaction conditions the PCR are as follows, 94° C for 2 minutes, followed by 35 cycles of 94° C for 30 seconds, 62° C for 30 seconds, and 72° C for 30 seconds. A final extension step of 72° C for 5 minutes concluded the cycle.

The majority of the seed lots examined in this study were mostly lots sold for human consumption. Seeds from 6 countries were analyzed in these studies, with the majority of seeds being sourced from Bolivia. Thirty-two seed lots were analyzed in this study and all lots along with country of origin are listed in the results section (Table 4-1).

Detection in Quinoa Plant Tissue

Quinoa foliar tissue known to be infected with *P. variabilis* was collected from field and greenhouse experiments and DNA was extracted as previously described. The PCR conditions for amplification were the same as were used for the primer based detection method with seeds.

To determine if *P. variabilis* could be detected in quinoa seedlings grown from infected quinoa seeds, seedlings were grown for two weeks in pasteurized soil, then seedlings were harvested and dried overnight at 55° C. Fifty dried seedlings for each seed lot were ground to a powder and 0.02 g of homogenized sample was used for DNA extractions. DNA was extracted as previously described. DNA was amplified using two consecutive PCR cycles in which the product of the first reaction served as the template for the second reaction. Both reactions used primer pair PV6 and the reaction conditions that are described in the previous section. PCR products were run on an agarose gel and stained with ethidium bromide for detection. Products that produced bands were sequenced to confirm the presence of *P. variabilis*.

<u>Phylogeny of the Causal Agent of Quinoa Downy Mildew in South America</u>

Samples of quinoa leaves with symptoms of quinoa downy mildew were collected in Bolivia and Ecuador by collaborators (see figure 4-1 for locations). The samples were dried and shipped to Penn State. For the Bolivian samples, there were 19 collection sites. For each site, 5 leaves (A-E) were chosen and a single lesion picked from the leaf for DNA extraction. For Ecuador, 78 samples were sent, but samples were degraded upon arrival, so a single leaf or leaf portion was chosen for DNA extraction. The Landisville and Rock Springs lines of *P. variabilis* were also included in these studies. DNA extractions, amplification and sequencing were performed as described in the previous section. For amplification, only the first PCR (using primers ITS4 and DC6) of the semi-nested PCR was used.

For phylogenetic analysis, obtained sequences were cleaned using ChromasLite (Technelysium Pty Ltd). Sequences were aligned using MUSCLE (Edgar, 2004) in MEGA5 and manually checked. For related *Peronospora* species and outgroups, the same sequences were

chosen that were used by Choi *et al*. (2010, (26)) in their study which reclassified the causal agent of quinoa downy mildew. A maximum likelihood tree was constructed in MEGA5 using the Kimura 2-parameter model with gamma distribution with invariant sites. The test of phylogeny used for this tree was the bootstrap method with 1,000 replications

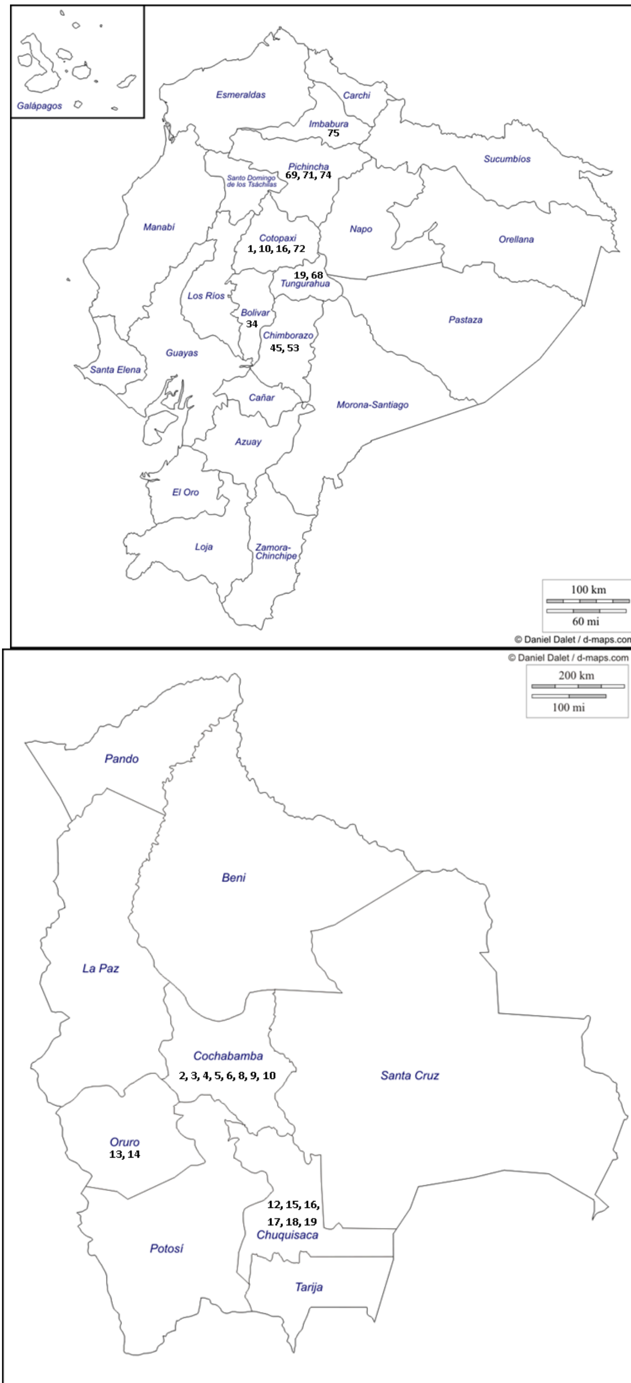


Figure 4-1 Locations of sample collection for *Peronospora variabilis* phylogenetic studies: These maps show the locations where quinoa downy mildew samples were collected.

Ecuador samples (ECDM#) were collected in the central Andean provinces of Ecuador and six provinces were represented. Samples ECDM16, ECDM19, ECDM68, ECDM69, ECDM71, and ECDM72 were wild *Chenopodium* species thought to harbor the quinoa downy mildew pathogen.

Bolivia samples (BDM#) were collected from three Bolivian departments and all samples were *Chenopodium quinoa*.

Biological Control of Quinoa Downy Mildew

Two trials were performed to determine if *Bacillus* could be used as a biological control agent. The two *Bacillus* strains used in these trials were *Bacillus mycoides* BmJ, a host defense inducer originally isolated from beet (9), and *Bacillus pumilus* GB34, a host defense inducer (71) from cucumbers.

Bacteria were grown overnight and adjusted to $\log_{10}$ 7-8 CFU/mL (first experiment, both 7.9, second experiment, BmJ: 7.2, GB34: 7.5). The first true leaf of 15-16 day old quinoa plants was syringe infiltrated with a suspension of one of the *Bacillus* strains or sterile water. One week later, the first leaf above the infiltrated leaf was sprayed with *P. variabilis* using an atomizer held six inches from the leaf. *P. variabilis* inoculum was prepared from sporulating leaves of the Landisville line and sporangia were shaken from the leaves in a mix of sterile water and 0.1% Tween 20. Sporangia were adjusted to 2.6-2.75 x 10^4 sporangia/mL. Plants were bagged for 24 hours. Bags were removed and plants were checked for symptom development for one week. Ratings of leaf area affected by *P. variabilis* were taken at one week post inoculation (35).

After one week, the *P. variabilis* infected leaves were removed from each test plant and incubated at 20° C on 1% water agar. After 24 hours incubation, a #6 cork borer was used to cut a circle from the most densely sporulating portion of the leaf and the plug was placed in 1 mL of sterile water in a microfuge tube. Tubes were vortexed for 30 seconds to remove sporangia and sporangia were counted using a hemocytometer. Sporangia from each leaf were counted six times in the hemocytometer to get a mean sporulation level for that leaf.

Results

Test of *Peronospora variabilis* Mating Types

Abundant oospores were detected in quinoa leaf tissue after a one week long incubation (Figure 4-2). This indicates that the Landisville and Rock Springs *P. variabilis* lines are different mating types. No oospores were observed in leaves infected with sporangia from a single *P. variabilis* line.

Figure 4-2 Oospores of *Peronospora variabilis* obtained by mating Landisville and Rock Springs *P. variabilis* lines in quinoa leaves.

Development of *Peronospora variabilis* Specific Primers

Out of 8 primer pairs tested, primer pair PV6 proved to produce the best results without amplifying DNA from the spinach downy mildew pathogen, *Peronospora farinosa* f.sp. *spinaciae*. Primer pair PV6 consisted of the following primers (both 5' to 3'): PV6F: GTTGCTGGTTGTGAAGGCTG and PV6R: ATGCTACGCAACCGAAGTCA. The ideal

annealing temperature was 62° C. Primer pair PV6 did not amplify DNA from any non-target organisms (Figure 4-3).

Molecular Detection of *Peronospora variabilis* in Quinoa Seeds and Plant Tissue

Methods to detect *P. variabilis* in quinoa seeds and plant tissue were developed in this research. Thirty-two seed lots were screened using these methods. The sequence based method of detection was able to detect *P. variabilis* in 25 of the seed lots (results shown in table 4-1). The PCR based detection method, with *P. variabilis* specific primers, was capable of detecting *P. variabilis* in 23 of 32 seed lots (Figure 4-3). When comparing the two methods, the PCR based method did not detect the pathogen in two samples that the sequencing based method was able to detect. This is likely due to the sequencing method being far more sensitive because it uses a semi-nested PCR method, so it can detect a lower copy number of pathogen DNA. No false positives occurred in the PCR based method of detection which is very important for developing reliable methods for seedborne pathogen detection.

For detection in plant tissue, 18 seed lots produced enough viable seeds for testing. Of the 18 seed lots, 10 tested positive for *P. variabilis* (Figure 4-4). Of the 10 seedling lots that tested positive, two (Faro2 and WMB) never tested positive in the molecular detection assays for seeds. The PCR based detection method always produced bands from infected leaf tissue.

Table 4-1 Results of molecular screening of quinoa seeds for *Peronospora variabilis:* Seed sources were molecularly tested for the presence of *Peronospora variabilis* using either sequencing or PCR with specific primer based methods. Results of the two screens are shown in the final two columns. Seeds produced in six different countries were tested.

Seed Source	**Country of Origin**	**Sequencing Confirmation of *P. Variabilis***	**Amplification of *P. variabilis* with specific primers**
Alter Eco Pearl (AEP)	Bolivia	Present	Yes
Alter Eco Red (AER)	Bolivia	Present	Yes
Alter Eco Mixed (AEM)	Bolivia	Present	Yes
Wegman's Bulk White (WegW)	Bolivia	Present	Yes
Wegman's Bulk Tricolor (WBT)	Bolivia	Present	Yes
Wegman's Bulk Red (WBR)	Bolivia	Absent	No
Wegman's Red (WegR)	Bolivia	Present	Yes
Wegman's Bulk Black (WegB)	Bolivia?	Present	Yes
Eden Organic Red (EOR)	Bolivia	Present	Yes
Eden Organic White (EOW)	Ecuador	Present	Yes
Arrowhead Mills White (AHMW)	Bolivia?	Present	Yes
IC Norte Granos Quinoa Real (ICN)	Bolivia	Present	Yes
Productos Cocinero Quinua Real (COC)	Bolivia	Present	Yes
Block S.R.L. Quinoa Real (BLO)	Bolivia	Present	Yes
Earthly Choice White (ECW)	Bolivia	Present	Yes
Ancient Harvest White (AHW)	Bolivia	Present	Yes
Giant Bulk White (GIA)	Bolivia?	Present	Yes
Nature's Pantry Bulk (NPB)	Bolivia?	Present	Yes
Kañiwa (KAN)	Bolivia?	Absent	No
Sahadi White (SAH)	Peru	Present	Yes
Phoebe White (PHW)	Peru	Present	Yes
Wheat Grass Kits Quinoa (WGS)	India	Present	Yes
Siskiyou '10 'Faro' (SIS10)	USA	Absent	No
Siskiyou '11 'Faro' (SIS11)	USA	Present	Yes
White Mountain White (WMW)	USA	Present	Yes
White Mountain Black (WMB)	USA	Absent	No
Greenhouse 'Faro' (F1, F2)	USA	Present/Absent	No/No
Chenopodium album (CAL)	USA	Absent	No
Norquin Golden	Canada	Present	No
Norquin Black	Canada	Absent	No
Norquin Golden Organic	Canada	Present	No
Norquin Black Organic	Canada	Present	Yes

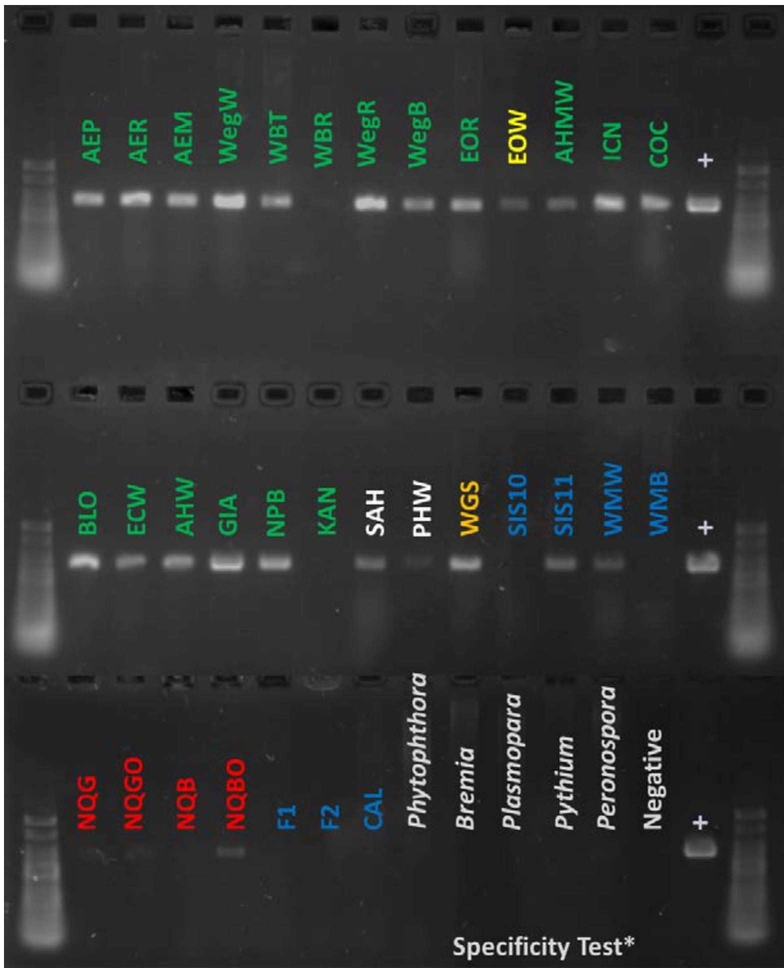


Figure 4-3 Results of gel electrophoresis of PCR products of a reaction using primer pair PV6 to selectively amplify *Peronospora variabilis* from quinoa seed washes. Samples are color coded by country and sample codes can be found in Table 4-1. The final 6 samples in the fourth row are a specificity check for the PV6 primer set. Primer set PV6 did not amplify DNA from *Phytophthora infestans* on tomato, *Bremia lactucae* on lettuce, *Plasmopara obducens* on impatiens, *Pythium aphanidermatum* or *Peronospora farinosa* f. sp. *spinaciae* on spinach. Seed lot codes can be found in Table 4-1.

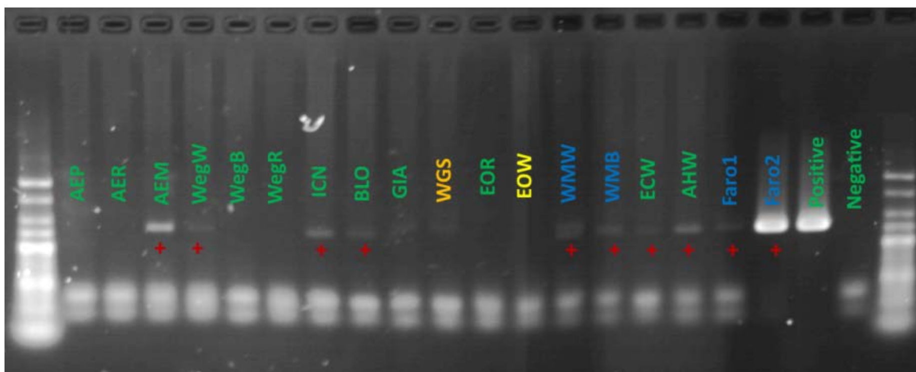


Figure 4-4 Amplification of *P. variabilis* DNA from quinoa seedlings grown from infected seed lots. DNA was amplified using primer set PV6 in two sequential reactions.

Phylogeny of the Causal Agent of Quinoa Downy Mildew in South America

ITS sequences were collected from quinoa downy mildew samples from the United States (3 sequences), Ecuador (12 sequences) and Bolivia (54 sequences). There were many Ecuadorean and Bolivian samples from which sequences could not be obtained.

The phylogenetic tree (Figure 4-5) produced in this study demonstrates that the United States, Ecuadorean and Bolivian quinoa downy mildew samples all were infected with the same species, *P. variabilis*. Secondly, ITS sequences obtained from weedy *Chenopodium* species in Ecuador also appear to be *P. variabilis*. This indicates that other *Chenopodium* species could serve as hosts for *P. variabilis*, but further studies are needed to confirm if this occurs under natural settings. It should also be highlighted that the causal agent of quinoa downy mildew is distinct from the causal agent of spinach downy mildew (samples SPDM1 and SPDM3). These two causal agents used to be classified as formae speciales of *Peronospora farinosa*, but this study supports, based on the ITS region, there two are separate species (26).

Biological Control of Quinoa Downy Mildew

Sporulation rates (sporangia/leaf disk) did not significantly differ by treatment in either the first (p=0.368) or second trial (p=0.214). However, in both trials, a reduction in sporulation was observed for both GB34 and BmJ treatments compared to the control (Figure 4-6). The leaf area affected by *P. variabilis* was also not significant for either experiment (*data not shown)*.

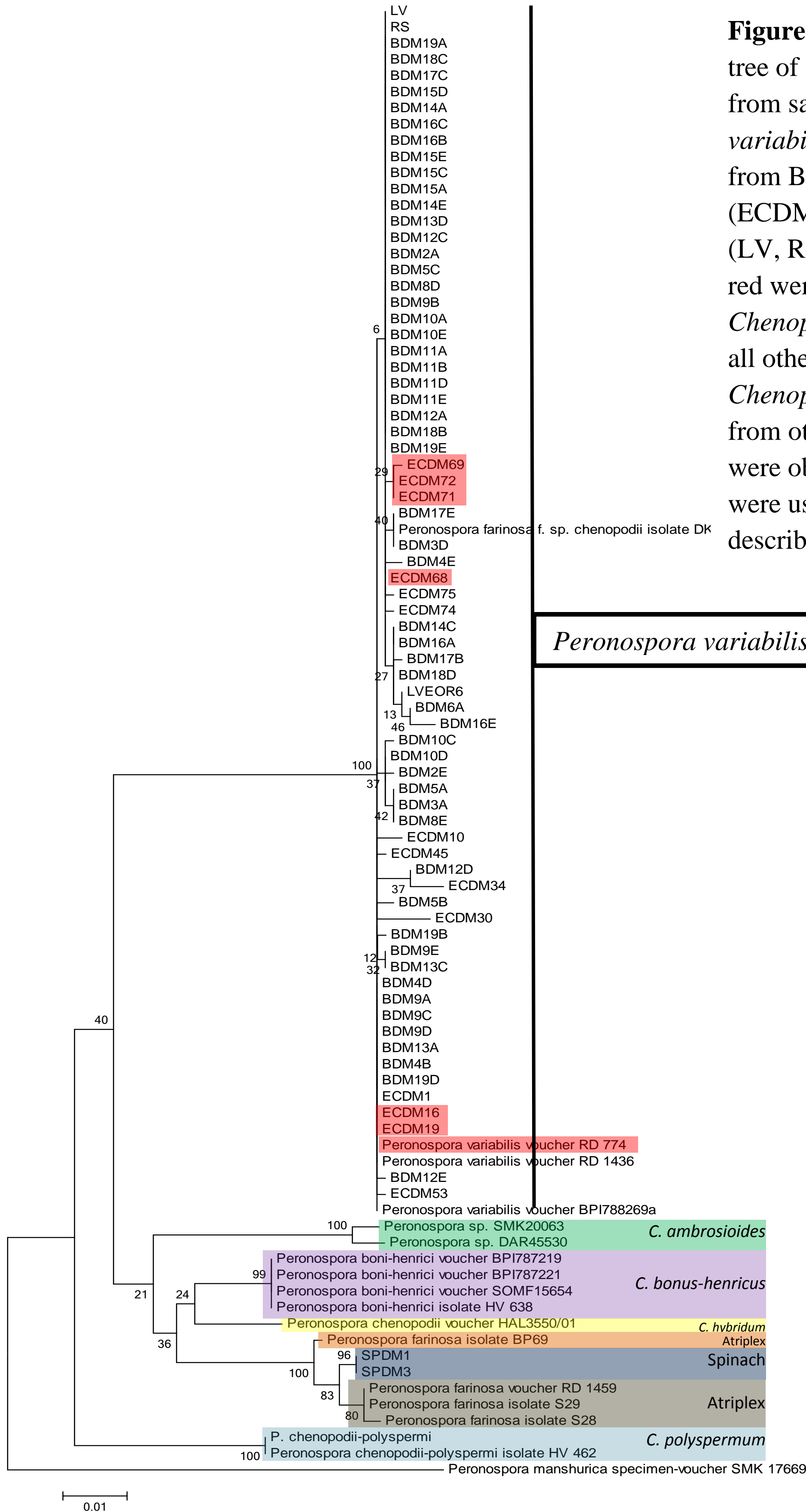


Figure 4-5 Maximum likelihood tree of ITS sequences obtained from samples of *Peronospora variabilis*. Samples were obtained from Bolivia (BDM), Ecuador (ECDM) and the United States (LV, RS, LVEOR6). Samples in red were collected from wild *Chenopodium* weed species, while all other samples were from *Chenopodium quinoa*. Sequences from other *Chenopodium* species were obtained from GenBank and were used as outgroups as described by Choi, et al. (2010).

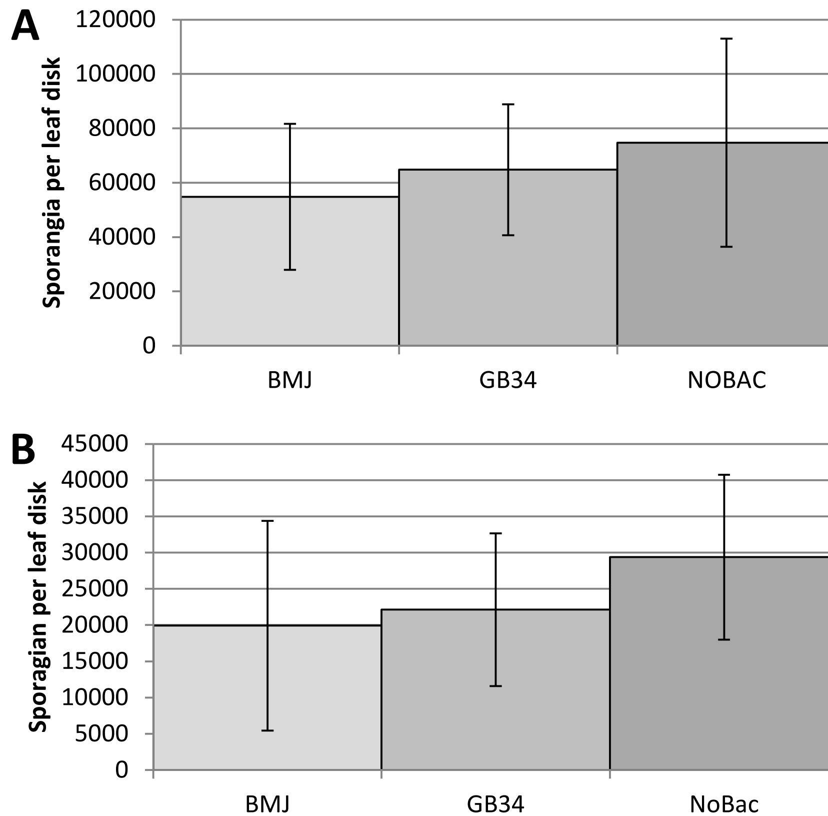


Figure 4-6 Sporulation rates of *Peronospora variabilis* in biological control assays. Plants were treated with *Bacillus mycoides* BmJ, *Bacillus pumilus* GB34 or no bacteria and inoculated one week later with *P. variabilis*. Inoculated leaves were harvested one week later and incubated on water agar. Sporangia were counted after a 24-hour incubation. The excised leaf disk was approximately 0.79 mm^2.

Error bars are standard deviation.

Discussion

The discovery of quinoa downy mildew in Pennsylvania led to the research in this chapter. The original source of inoculum for the outbreak of quinoa downy mildew in Pennsylvania was unknown, so several experiments were designed to address this issue. Two potential inoculum sources were infected weedy *Chenopodium* species or infected quinoa seeds (imported, consumable seeds) used to plant the fields.

A molecular method for detecting *P. variabilis* in quinoa seeds was developed to determine if imported, consumable quinoa seeds could harbor the pathogen. *P. variabilis* was detected in nearly all seed lots of quinoa, and this highlights a critical inoculum source for management. Research into the use of fungicides or seed heating treatments to kill seedborne *P.*

variabilis is required to limit spread of quinoa downy mildew. Further, the molecular detection methods developed in this research are the first steps to creating certified *P. variabilis*-free quinoa seed.

P. variabilis was also detected in quinoa seedlings that were grown from infected seed lots. This suggests, but does not prove, that seedborne oospores are still viable. Therefore, seedborne oospores could have served as the source of initial inoculum for quinoa downy mildew in Pennsylvania. Oospores were observed in quinoa leaves collected at Rock Springs and the two *P. variabilis* lines are capable of mating and forming oospores, so this suggests that there were multiple viable oospores that led to initial infections at both sites.

To determine if weedy *Chenopodium* species could harbor *P. variabilis*, it first had to be determined if the pathogen on the weeds was the same pathogen that infects quinoa. From phylogenetic analysis of quinoa downy mildew samples from South America, it was determined that the species of *Peronospora* that infects weedy *Chenopodium* species is *P. variabilis*, based on the ITS region. Based on studies performed at Penn State, *Chenopodium album* collected from the Russell E Larson Research Farm was not susceptible to *P. variabilis*, although *C. album* has been reported to be susceptible to *P. variabilis (38)*. *Chenopodium pallidicaule*, the Andean grain crop cañihua (kañiwa), was susceptible to *P. variabilis* isolates collected in the United States. This indicates that while the Pennsylvania native population of *Chenopodium* was not susceptible to *P. variabilis*, the isolates obtained in the United States were still capable of infecting *Chenopodium* species more closely related to quinoa, mainly cañihua. In regards to the Pennsylvania outbreak of quinoa downy mildew, this provides further evidence that the primary inoculum was seedborne and not from other *Chenopodium* species.

Quinoa downy mildew is the most economically important disease of quinoa. In order to maintain quinoa yields, better disease management practices must be developed for quinoa downy mildew. In this research, a rapid, reliable method for detecting *Peronospora variabilis* in quinoa seeds and plant tissue was developed. The phylogeny of *Peronospora variabilis* in South America was examined and it was concluded that, in South America, the pathogen that infects weedy *Chenopodium* is that same species that infects quinoa. Further research is required to determine if these weedy *Chenopodium* are actually contributing inoculum to quinoa downy mildew epidemics. The conclusions that were drawn from these studies are a step towards developing sustainable disease management strategies for quinoa downy mildew.

Chapter 5

International Experiences: Developing a Program to Teach IPM in Tiraque Province, Bolivia and Collaborative Research Experiences with Andean Researchers

Introduction

This chapter consists of two parts: 1) an extension program designed to teach Andean farmers about IPM and 2) a compilation of materials developed for collaboration with Bolivian and Ecuadorean collaborators. The mock program was developed as part of a project for AEE450: Program Design and Delivery.

Part I: Extension Program to Teach Andean Farmers about Integrated Pest Management of Quinoa Downy Mildew

Introduction

Quinoa, *Chenopodium quinoa*, is a culturally, economically and historically important crop in the Andean region of South America (31). Bolivia is one of the largest exporters of quinoa, exporting mainly to rapidly expanding markets in North America and Europe. As international demand for quinoa increases, farmers receive more income from the crop, but traditional cropping systems become strained and unsustainable. With increased profit, Andean farmers can now afford more crop inputs, such as fertilizer and pesticides. However, because these inputs have historically not been used in Andean farming, farmers need to be educated on how to sustainably utilize these resources.

The focus of my thesis research is identifying *Bacillus* isolates that could be used as biofertilizers and/or biological control agents. The program described in this paper is a theoretical program that would act as a technology transfer of a microbial biopesticide to Andean

farmers. This program could also be adapted to incorporate synthetic pesticides, but this is less favorable due to demand for organic quinoa, the cost of synthetic pesticides, environmental non-target effects, and the potential development of resistance to the pesticides.

In this program, Andean farmers will learn about the key disease of quinoa, quinoa downy mildew, caused by the oomycete pathogen, *Peronospora variabilis*. Quinoa downy mildew leads to severe yield reduction and reduced seed quality if left unchecked (35). Quinoa downy mildew can spread rapidly and must be carefully managed to reduce disease severity and incidence. Management of quinoa downy mildew can be worked into an integrated pest management (IPM) framework. In IPM, multiple tactics for pest control are combined to save money, reduce environmental impacts and improve human welfare. In this program, participants will learn about quinoa downy mildew, biopesticide application and integrated pest management of quinoa downy mildew.

Location

This program will be held in the town of Tiraque, Bolivia. Tiraque is located about 60 kilometers from Cochabamba, a major Bolivian city. Tiraque is the main city of Tiraque Province. Uzeda (2005, (128)) compiled a great deal of information on the population of Tiraque from 2001 census data and other sources. The 2001 population of Tiraque Province was 35,000, of which approximately 31,000 were rural. A 1992 study of poverty in Bolivia found that 97% of Tiraque Province's population could be classified as poor (128).

Agriculture is the major occupation in Tiraque Province and is worth around $8 million annually (128). The major crops grown in Tiraque Province are potatoes (Solanum tuberosum), quinoa, faba bean (*Vicia faba)*, onion (*Allium cepa*), wheat (*Triticum aestivum*), pea (*Pisum sativum*), and maize (*Zea mays*) (128). Other traditional Andean crops such as oca (*Oxalis*

tuberosa) and tarwi (*Lupinus mutabilis*) are also grown in Tiraque Province (personal observation, 2011). Herding and livestock production are also important on the highly diversified farms found in Tiraque Province.

Collaborators and Target Audience

The collaborators for this project are members of the PROINPA (Promotion and Investigation of Andean Products) Foundation. PROINPA is a non-governmental organization with its headquarters in Cochabamba, Bolivia and regional offices across Bolivia. PROINPA focuses on all aspects of agricultural research, from basic agricultural science research to social science studies of agriculture. PROINPA also functions as an extension agency, with extension agents performing research and demonstration trials in local farmers' fields and advising farmers on best practices (Testen, personal observation, 2011). PROINPA extension agents would be ideally suited to advertise and deliver the program in Tiraque.

The target audience for this program is poor Andean farmers located in Tiraque Province. Farmers that grow quinoa for both sustenance and export will be recruited to participate in this program. Because some of these farmers are so remote, farmers that have contact with PROINPA extension agents are most likely to learn about this program and attend it. Farmers that would benefit from this program could also be identified by talking to heads of the peasant unions of Tiraque Province (128). The peasant unions typically consist of land-holding farmers, so this may disadvantage farmers that do not own land from attending the program.

Program Objectives

Quinoa Downy Mildew Objectives

-90% of participants will identify quinoa downy mildew symptoms versus other diseases of quinoa as measured by an identification activity during the program.

-75% of participants will be able to identify the proper stage of quinoa downy mildew at which they should apply bio-pesticides by the end of the program.

Biopesticide Application Objectives

-75% of participants will demonstrate how to calibrate and use a backpack sprayer to apply bio-pesticides when visited 3-6 months later by an extension agent.

-75% of participants will demonstrate how to prepare and apply *Bacillus* bio-pesticides when the bio-pesticides are delivered by extension agents 1-3 months after the program.

Integrated Pest Management Objectives

-By the end of the program, 85% of participants will be able to identify at least 3 alternative practices that can be used to reduce quinoa downy mildew as measured by a spoken survey.

-Within one year, participants will use at least two IPM practices to reduce quinoa downy mildew as measured by an interview with an extension agent.

Curriculum Guide

This program will be presented as a field day combined with several workshops. This format was chosen instead of a farmer field school program, because the present program seeks to educate more people for less money. Bentley (2007, (12)) found that farmer field schools were most effective for teaching Bolivian farmers about bacterial wilt of potato, but also found that community workshops were effective for transmitting the same information for less money. With this program format, more farmers can be reached for less money and time input. Our format also allows for information to be transmitted in a variety of ways, which can improve comprehension and retention.

The program will be delivered in Spanish, Quechua, and Aymara. Spanish and Quechua will be the main languages spoken and there will be Aymara translators on hand if necessary.

Quechua will be the main language used because approximately 75% of the population of Tiraque Province speaks Quechua (128). The handouts on spraying methods, bio-pesticide preparation, and spray timing will be mostly visual with some clarifying remarks written in both Spanish and Quechua. The literacy rate in Tiraque Province is around 78%, so it is appropriate to include some written program handouts (128).

The program will be held on a weekend in the town of Tiraque. The town of Tiraque is a social and economic hub of Tiraque Province, so most individuals in Tiraque Province have means of reaching the town of Tiraque. There is a major Friday market in the town of Tiraque and individuals could stay in town for the program after the market. Also, the program will be repeated on both Saturday and Sunday, so participants may be able to attend on either day. Participants may also be in the town of Tiraque to go to church services on Sunday.

Field Day

The field day portion of the program is to demonstrate IPM practices under actual field conditions. Cultural practices such as crop rotations, plant spacing and foliage thinning will be demonstrated in plots to show farmers how they can reduce quinoa downy mildew without pesticides. There will also be plots to demonstrate the effectiveness of *Bacillus* bio-pesticides against quinoa downy mildew. These plots will be staffed by PROINPA extension agents to describe the treatments and also to answer any questions participants may have about the plots.

Demonstrations

There will be two key demonstrations related to bio-pesticide application. These demonstrations will be set up near the field trials, so that participants can try the activities for themselves in the field. The first demonstration will be how to properly prepare bio-pesticides for application. The activity will be demonstrated by a PROINPA extension agent and

participants can practice the preparation with just a talc powder to simulate the final *Bacillus* product. By letting the participants watch and try they technique themselves, the participants will retain more information.

The second demonstration will be of how to use a backpack sprayer. Again, a PROINPA extension agent will demonstrate the basics of calibration and spraying, and participants can try it for themselves. Several models of backpack sprayers will be available to teach the differences between models. Farmers will also be introduced to protective measures and clothing that should be worn when spraying bio-pesticides and other pesticides.

Workshops

There will be three workshops during this program. The first workshop will be a workshop on quinoa downy mildew identification. This is one of the most important workshops because if you cannot properly identify the disease you're working with, the disease will not be properly treated. There will be plants infected with quinoa downy mildew at different stages of growth and development. There will also be plants with other diseases and nutrient deficiencies to compare to quinoa downy mildew symptoms. Participants will be asked to correctly identify plants with quinoa downy mildew when mixed with plants with other diseases to show that they learned how to correctly identify the symptoms.

The second workshop will be on spray timing for spraying bio-pesticides at times when they would be most effective. Because quinoa downy mildew is a disease that spreads very rapidly, timing of bio-pesticide sprays is critical. Participants will learn how to identify the different stages of disease development. A simple consistent method developed from field research will be used (36). Participants will also learn at what stages of disease development the

plant could be considered a loss and should be rogued from the field. Simple charts will be given to participants to take with them to reference when to spray and when not to spray.

The final workshop used in this program will be a participatory workshop on IPM techniques that participants can use to combat quinoa downy mildew with reduced input from pesticides. A brief overview of basic IPM concepts will be introduced to begin the workshop. Next, participants will discuss which methods they already use to combat quinoa downy mildew. Some of these methods could be beneficial for other farmers to adopt but some practices may not have an effect on quinoa downy mildew. The ineffective practices will be discussed and it will be explained why they do not help reduce disease. The suggestions of the farmers will be compiled on a big sheet of paper or blackboard for all to see. The ideas will also be verbally repeated, so that participants that cannot read can still participate in the discussion.

Workshop leaders (PROINPA extension agents) will introduce other IPM techniques to use that farmers may not have used in the past. Examples of three IPM techniques would be better field sanitation, using resistant varieties, and rotation. It will be explained why these techniques would reduce quinoa downy mildew. Sanitation reduces the number of spores that can infect other plants, whereas using resistant quinoa varieties can reduce disease overall. Finally, introducing rotation is very important because the quinoa downy mildew pathogen can overwinter in soil and plant debris. Quinoa is being continuously cropped in some areas due to the high prices it receives, but this is not good for long term crop and soil sustainability and disease reduction. The use of bio-pesticides in quinoa downy mildew reduction will also be discussed extensively in this workshop.

Sub-program for Children

While the adults are learning about quinoa downy mildew IPM, there will be a separate, fun program for children. This program will focus on quinoa consumption because malnutrition has increased as quinoa consumption has decreased in favor of more palatable processed starches such as bread or rice (105). The objective for this program is that 85% of children will increase their knowledge about quinoa nutrition and preparation by the end of the workshop as measured by pre- and post- surveys. This activity is meant to be fun and entertaining and will also have educational games for the children to play. There will also be hands-on activities on quinoa preparation and there will be quinoa based foods for participants to try. This program would hopefully increase interest in quinoa consumption in children, also. However, because this is only a small subset of the overall program, only the simple pre- and post-program surveys will be given to evaluate if knowledge of quinoa was increased.

Community Member Involvement

Collaboration with PROINPA is essential for the success of this program. Because PROINPA is local, they have resources and knowledge of Tiraque that no other organization would have. PROINPA has scientists that research quinoa downy mildew and sustainable methods to control the disease. PROINPA also has extension agents and social scientists that can help develop and deliver the program on IPM of quinoa downy mildew. The responsibilities of PROINPA are to be the "boots on the ground." PROINPA will be the main deliverers of the program, although individuals from Penn State could travel to Bolivia to assist with the program. PROINPA has individuals that speak Spanish, Quechua and Aymara, so the program can be delivered in any language necessary.

PROINPA will work with local farmers to use their fields to create demonstration plots for the field day portion of the program. The farmers chosen for this portion of the program will

need to be very close to the town of Tiraque, so that program participants can easily reach their fields. These farmers will be monetarily compensated for the use of their land, but will also learn about IPM from demonstrations in their fields.

During the program, it is important that participants get the opportunity to discuss techniques that they use besides synthetic or biological pesticides to combat quinoa downy mildew. By taking a participatory approach to discussing IPM of quinoa downy mildew, more opinions of the farmers are heard. Participation by the farmers can lead to new ideas coming out that work in the IPM framework that scientists may have overlooked. This inclusion of indigenous knowledge can greatly strengthen the program. Also, through participation and discussion, common misconceptions can be discussed, so that farmers learn together with the programmers what needs to be corrected.

Timeline

Time	Activity	Who does it?
October Year 1	Identify famers to invite to program Identify fields to use in field demonstrations	PROINPA
October-March Year 1	Prepare program materials Arrange facilities in Tiraque Prepare some field demonstrations	Penn State, PROINPA
August Year 1	Hold weekend program Pre and post-surveys of quinoa downy mildew and IPM knowledge	PROINPA, Penn State
September-October Year 1	Deliver bio-pesticides Check backpack sprayer skills Short-term follow-up	PROINPA
October-March Year 2	Long-term follow-up Evaluate if IPM practices are being implemented	PROINPA, Penn State
March and beyond Year 2	Gather all evaluation data Rework program elements that need improvement	PROINPA, Penn State

	Expand program to other quinoa producing regions of Bolivia if successful	

Advertising the Program

This program would be advertised in several settings and in several ways. First, advertisements for the program would be broadcast on local radio stations. PROINPA extension agents would spread word about the program as they visit different farmers. There is also a large Friday market in the town of Tiraque on Fridays and this would provide a perfect opportunity to inform farmers of the upcoming program. By advertising at the Friday market, farmers that are already be able to travel to Tiraque would learn about the program. At the Friday market, the advertisement would be both visual, through handouts and posters, and verbal, through announcing it throughout the market. The heads of the peasant unions of Tiraque Province will also be notified about the program so that they can tell their members about it. By having multiple methods of advertising the program, more individuals will learn about this opportunity.

Evaluation Process

The evaluations used in the program are varied and should collect a great deal of information (98). The evaluations will be carried out by PROINPA extension agents, but it is possible that Penn State collaborators could be present at one of the short or long term evaluations in which individual farmers are visited.

The program will be evaluated at three different time points. During the program, participants will be asked about what they think of the program so far. This will provide informal opinions on the program and could also be used to make immediate changes to the program if simple problems are brought to the programmers' attention. A pre- and post-program

survey of farmers' knowledge of IPM and quinoa downy mildew (48; 114) and their impressions of the program will be used to measure knowledge and skills.

The second evaluation will be performed 1-2 months after the program when PROINPA extension agents deliver the bio-pesticides. At this evaluation, farmers will demonstrate how to use a backpack sprayer to spray bio-pesticides. This evaluation is to measure knowledge and skill retention. Evaluators will also note if the farmers have implemented any IPM tactics to determine if there has been a practice or behavior change.

The final evaluation will be performed the next season, one year after the program. In this final evaluation, farmers will be surveyed and fields examined for the implementation of IPM practices. Since one of the objectives is that farmers adopt at least two IPM techniques, this evaluation will determine if this objective has been achieved. This final evaluation will also tell us if farmers have changed their farming practices and behaviors to fit into the IPM framework that was proposed during the program. In this final evaluation, data can also be gathered on how much information the farmer has retained from the program, but it is most important to focus on if and how that knowledge is put to use.

Tracking Forms

	Program Planning, Development and Preparation
☐	Contact collaborators in Bolivia to discuss initial program plans
☐	Skype meetings of PROINPA and Penn State program developers
☐	Physical meeting of Penn State and PROINPA collaborators in Bolivia
☐	PROINPA identifies farmers to invite to program
☐	Contact farmers near town of Tiraque to lend fields to be used in the field day
☐	Plant and apply treatments to field demonstrations
☐	Prepare curriculum materials
☐	Secure space in the town of Tiraque to hold workshops and demonstrations
☐	Prepare handouts for farmers to reference on how to prepare bio-pesticides, how to use a backpack sprayer and how to identify when to spray
☐	Identify who will deliver the program
☐	Prepare living plant samples to demonstrate different diseases of quinoa
☐	Advertise program on the radio

☐	Inform peasant unions of program
☐	PROINPA extension agents invite farmers to program
☐	Advertise program at Friday market in Tiraque
	Program Delivery
☐	Hold workshop on spray timing
☐	Hold workshop on quinoa downy mildew identification
☐	Hold field day IPM technique demonstrations
☐	Hold participatory workshop on IPM techniques
☐	Workshop on how to prepare bio-pesticides
☐	Run quinoa education program for children during adults' program (prepare games, food, fun hands-on quinoa activities)
☐	Deliver bio-pesticides and ensure farmer can prepare it properly
	Evaluation
☐	Develop evaluation surveys, short term and long term evaluation questions (be able to provide in both written and spoken versions)
☐	Informally ask farmers their opinion on the program
☐	Give pre-program survey on IPM and quinoa downy mildew knowledge
☐	Give post-program test on IPM and quinoa downy mildew knowledge
☐	Evaluate farmers' skill with backpack sprayers
☐	Evaluate incorporation of IPM techniques into quinoa production
☐	Gather all evaluation data (day of program evaluations, short term evaluations and long term evaluations) and examine it based on pre-determined statistical tests
	Program Refinement and Expansion
☐	Skype follow-up meeting of PROINPA and Penn State program developers
☐	Refine program based on findings of evaluation (this may mean changing timing of program, changing where the program is offered, change how course materials are delivered, modifying objectives of the program, etc.)
☐	Explore expanding program to other quinoa producing areas of Bolivia and potentially Peru and/or Ecuador
☐	Expand programs to other regions if funding and interest are available

Tips

-When doing international programs, it is useful to be conversational in the language of the country. When working in areas with multiple languages, learn key words and phrases of the less common languages.

-Ensure that field plots and diseased plant examples are ready to go for the program. If participants are unable to see these examples of IPM and quinoa diseases in person, they are less likely to accept IPM practices. Seeing is believing!

-Having a side program for children ensures that parents can focus on the programming presented on quinoa downy mildew. The children can also benefit from learning about quinoa and having some fun in the meantime.

-Initial meetings over Skype or another video-chat program can greatly accelerate program design because they allow for real-time discussion of ideas. Video-chat also allows programmers to pick up each other non-verbal communication, so difficult ideas can be communicated and discussed more easily.

-Make sure you have enough handouts for the farmers that will attend. Try to get a rough headcount for printing purposes, but always print extra because they can be handed out to farmers that were unable to attend.

-Make sure the schedule for the day is clear to all participants. Divide participants into groups and rotate them through workshops and field visits. Make sure you have enough staff so that participants can get more attention if they have questions. Small groups of participants are especially important for the participatory discussion portions of the program.

-Be ready for any weather conditions because there is a field component for this program!

Conclusion

A program on IPM of quinoa downy mildew would likely benefit rural farmers in Tiraque Province. Because many different formats will be used to deliver the content of this program, it is expected that comprehension and retention will be improved over just using a single method of delivery. The evaluation methods of this program are also diverse and seek to

measure both KASA (knowledge, awareness, skills, and attitude) and practice and behavior change, so we expect to get results that will help improve future programs of a similar nature.

Part II: Collaborative Research Experiences with Andean Researchers

SANREM Traineeship of Mayra Claros

During June 2012, Mayra Claros visited Penn State as part of a SANREM traineeship. Ms. Claros is a researcher for Fundacion PROINPA in Cochabamba, Bolivia and her research focuses on selecting microbes that are beneficial to different Andean crops. Ms. Claros' research covers areas such as rhizobia, mycorrhizae, and plant growth promoting bacteria (PGPB), such as *Bacillus*. During her traineeship, Ms. Claros wished to develop skills that would enhance her ability to screen and identify plant growth promoting bacteria.

Ms. Claros worked mainly with myself in the lab and we worked together to identify skills that would be useful to her. To enhance *Bacillus* production, Ms. Claros learned a technique to clean *Bacillus* endospores to remove germination inhibitors and an endospore stain to check the quality and status of endospores during endospore production. To enhance screening of putative PGPB, techniques to measure phosphate solubilization in liquid media, chitinase production, and indole acetic acid production were tested on Bolivian isolates. Ms. Claros and I screened 342 Bolivian quinoa *Bacillus* isolates (discussed in Chapter 3) for chitinase production and IAA production. The process to create rifampicin resistant mutants was also taught, so that Ms. Claros would be able to screen for *Bacillus* colonization more reliably. Finally, Ms. Claros learned methods for working with *Peronospora variabilis*, such as how to maintain the pathogen in detached quinoa leaves, how to standardize and inoculate *P. variabilis* in quinoa plants, and how to perform *Bacillus* infiltrations of quinoa leaves for *P. variabilis* biocontrol assays.

During her time at Penn State, Ms. Claros learned a great deal about plant growth promoting research. Likewise, Ms. Claros also benefitted from speaking with other faculty and graduate students in the department to learn more about their research. I learned a great deal from Ms. Claros and look forward to working with her in the future.

Protocols for Bolivian and Ecuadorean Researchers

The following protocols were developed for use by PROINPA and INIAP researchers after they had requested written protocols for certain techniques:

Maintaining *Peronospora variabilis* in detached quinoa leaves (35; 93))

Required Materials

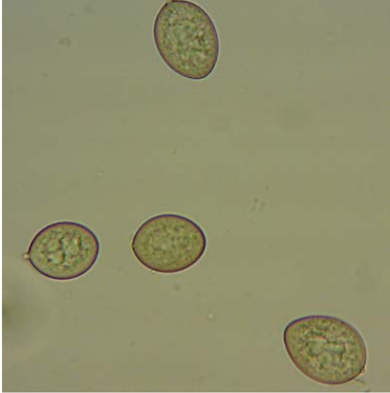
-Fresh *P. variabilis* sporangia
-Young quinoa leaves (plants 2-8 weeks old, leaves one inch in diameter or smaller)
-Pipettes
-Sterile water
-Petri dishes of 1% water agar
-Cell spreader
-15 or 50 mL conical tubes

1. Collect freshly sporulating leaves or take sporulating leaves from a fresh *P. variabilis* maintenance petri dish

2. Place 5-7 heavily sporulating leaves in a conical tube with 5 mL of sterile water. Shake or vortex the tube to remove the sporangia from the leaves.

3. Pour the sporangial suspension through 2-4 layers of cheesecloth into a new tube to remove leaf debris (you should get 2-4 mL of solution).

4. Check the sporangial solution under the microscope to check sporangia quality. Very hyaline sporangia are young and tan-brown sporangia are older.



5. Place 1 mL of sporangial suspension on 1% water agar and spread with a cell spreader.

6. Place young quinoa leaves onto the suspension on agar so that the top quinoa leaf is placed onto the agar. This way the sporangia will infect the top of the leaf and then sporulate from the bottom of the leaf where there are more stomata.

7. Place in paper bag or box for 20-30 hours at 20° C. Remove from bag and allow plates to incubate at 20° C with a 16 day, 8 hour night light cycle for 5-7 days until sporulation is visible on the leaves.

8. When leaves are visibly sporulating, repeat the maintenance process (Go to step 1).

Long term storage of *Peronospora variabilis*

The following methods can be used to store *P. variabilis* for at least six months.

Cryo-Storage in DMSO

1. Shake sporangia from leaves into 15% DMSO. Filter through cheesecloth and aliquot into cryo-tubes.
2. Place in -20° C freezer, allow to freeze and then freeze in liquid nitrogen. (Isolates can remain at -20° is liquid nitrogen is not available.)

Freezing sporulating leaves on water agar

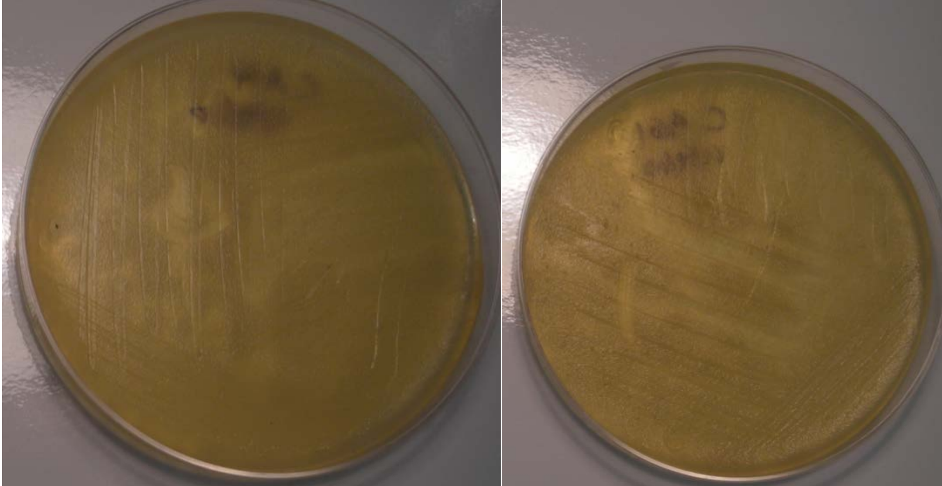
1. Take a plate of sporulating leaves on 1% water agar and triple parafilm to prevent water loss. Chill in refrigerator for approximately 1 hour and then place in a -20° C freezer.

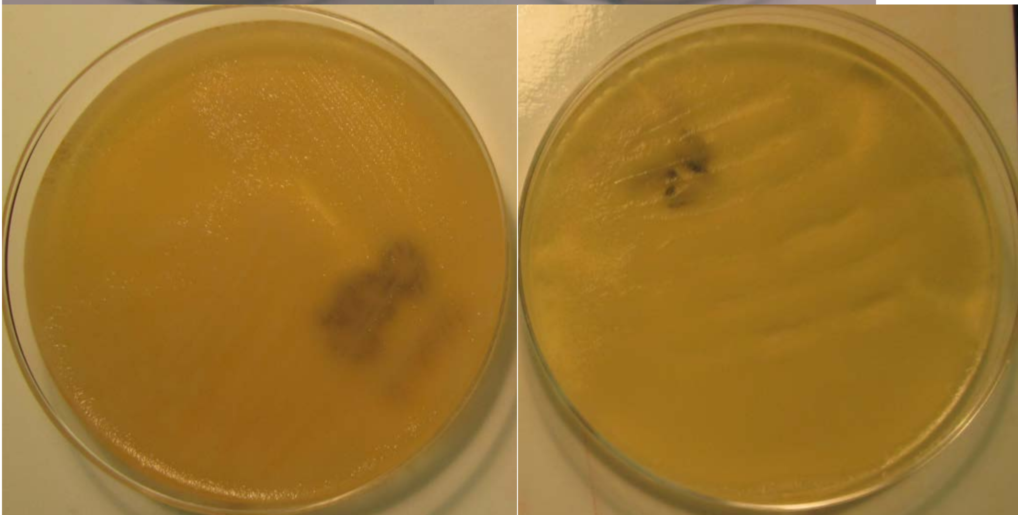
Freezing sporulating leaves on filter paper

1. Wrap sporulating leaf in moistened (dry also works, but not as well; very wet paper does not work very well) filter paper. Roll filter paper and place in 1.5-2 mL microfuge tubes in a -20° C freezer.

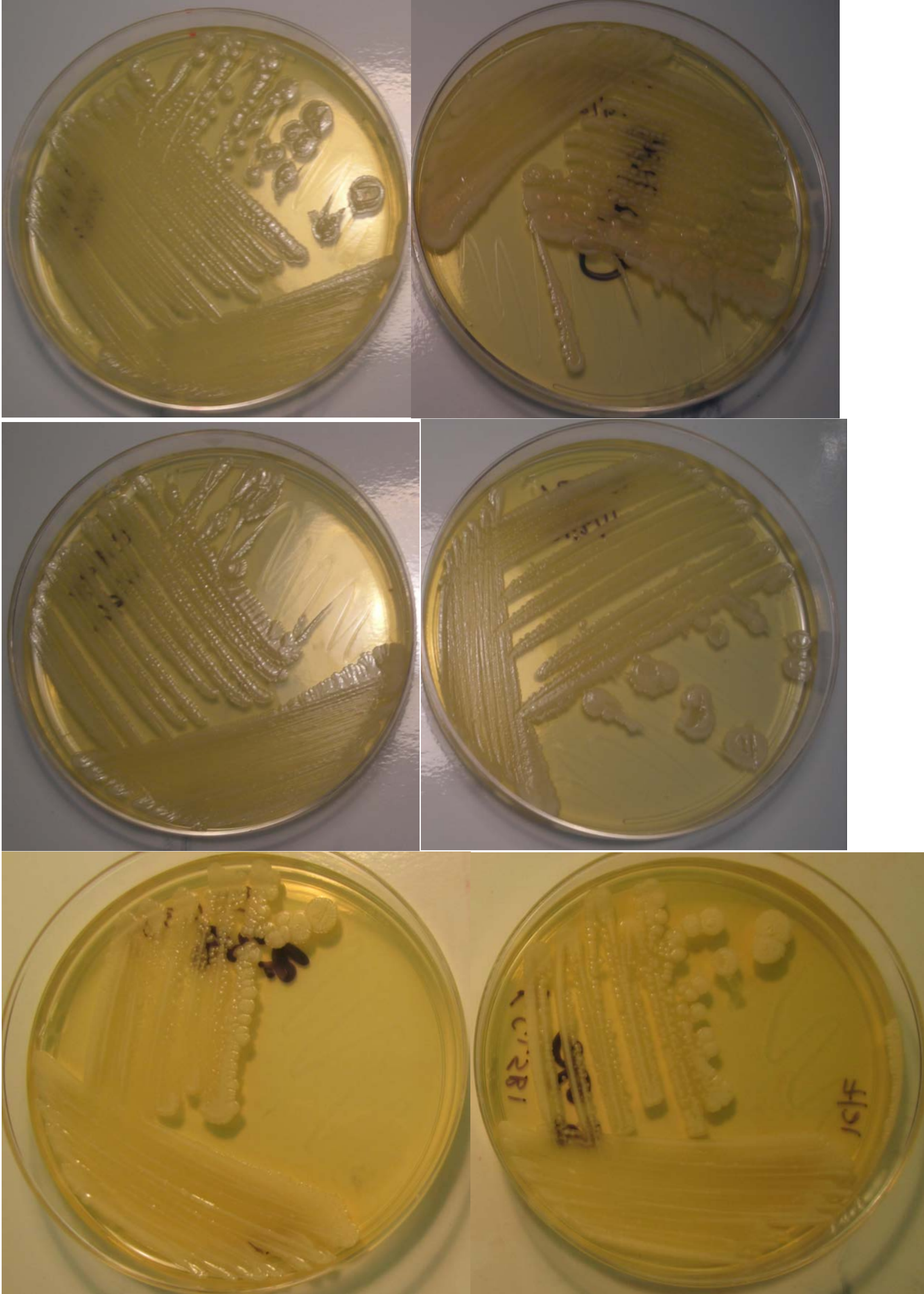
Bacillus Photo Guide
All cultures were grown on TSA and incubated for 24 hours at 30° C. Most of the cultures photographed are approximately 1 week old.

Bacillus mycoides

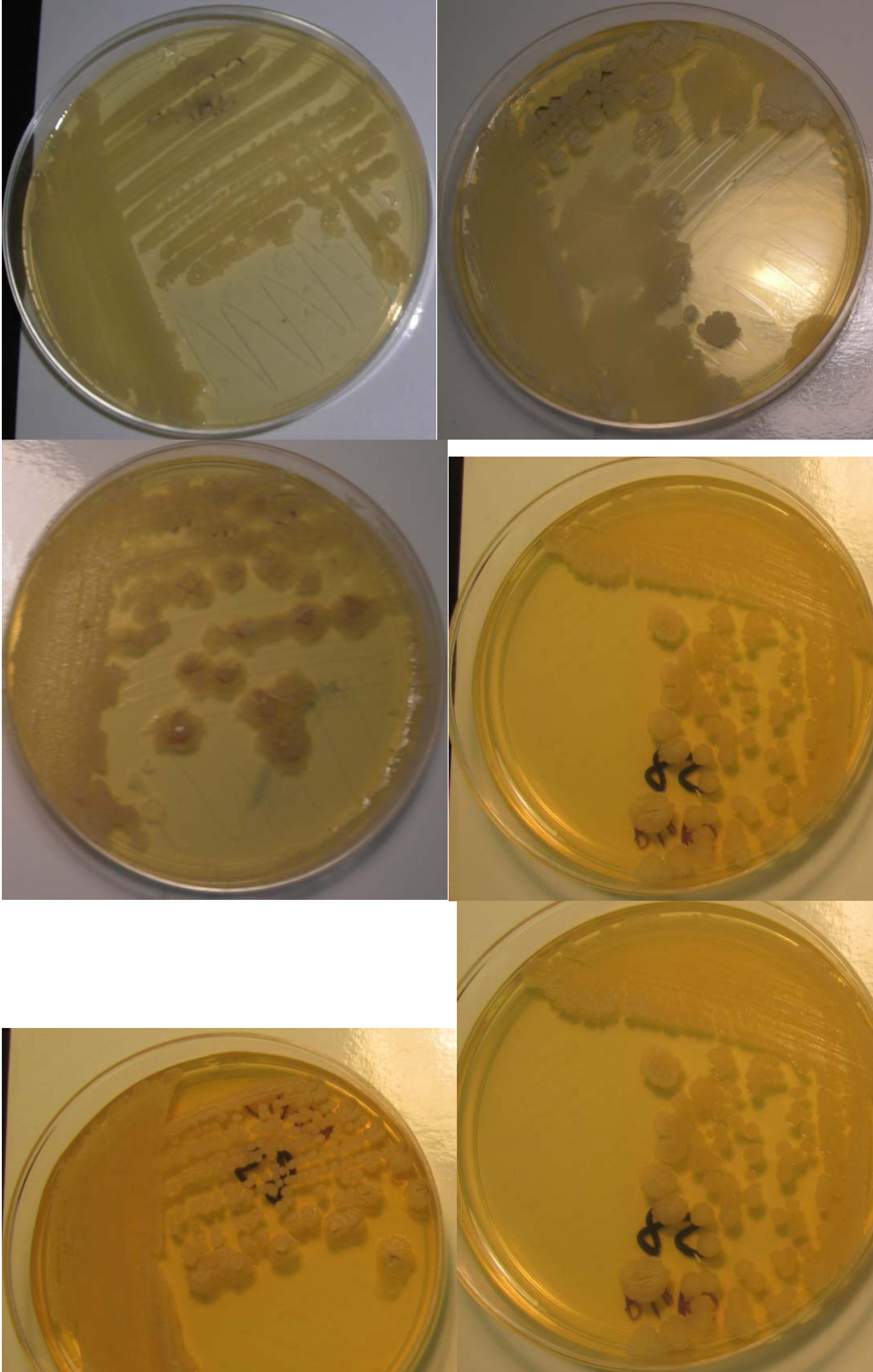




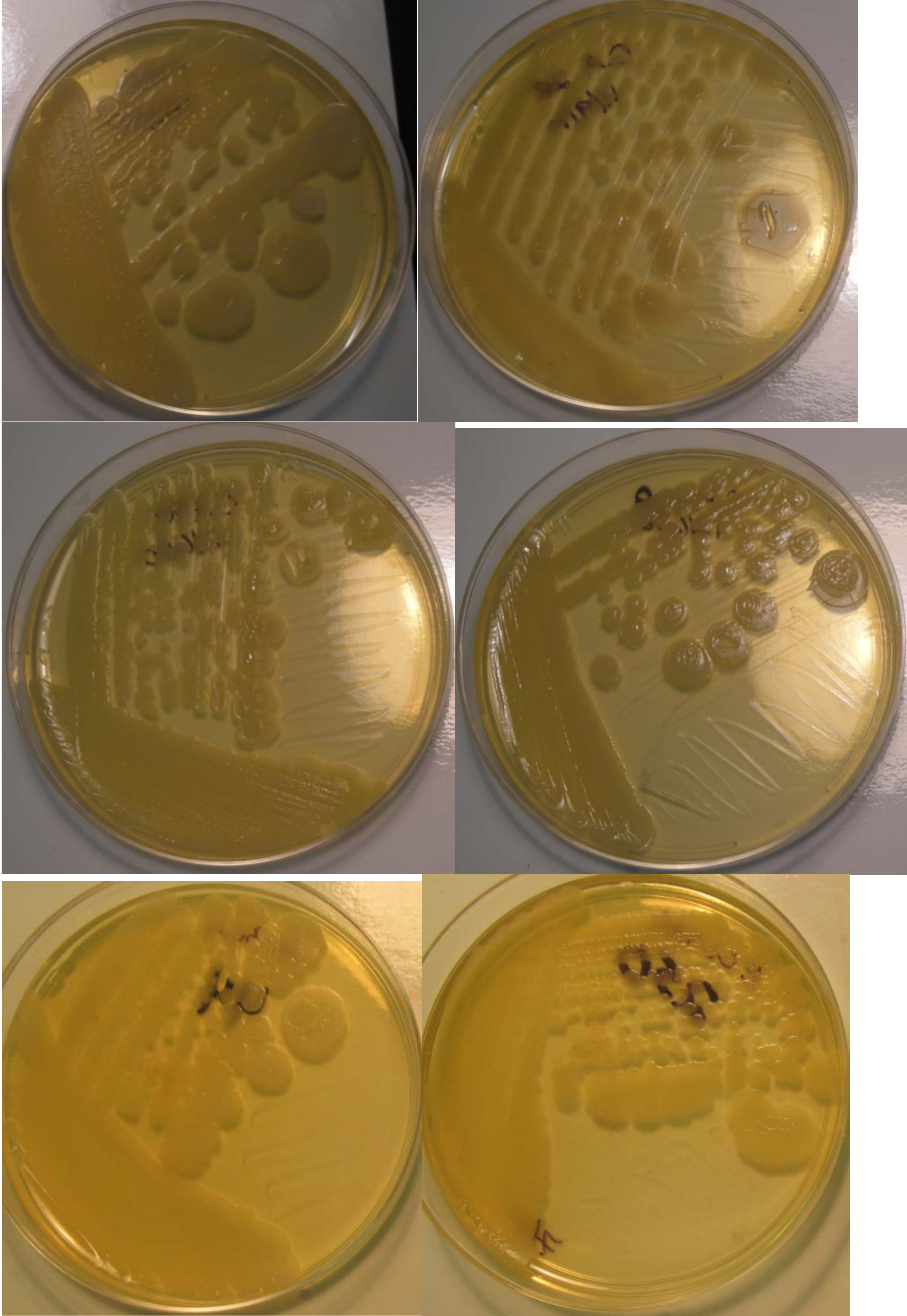
Bacillus pumilus



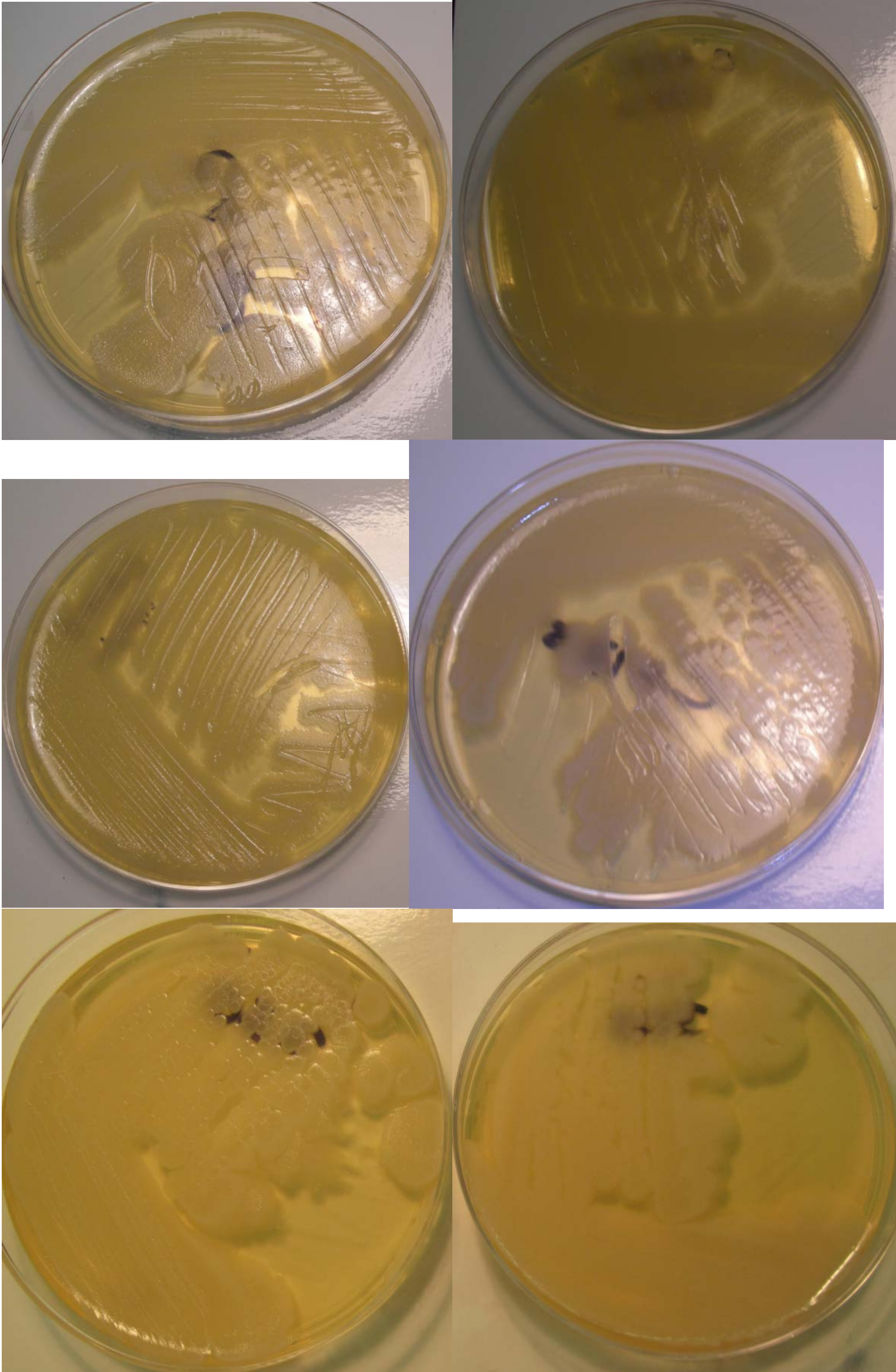
Bacillus subtilis



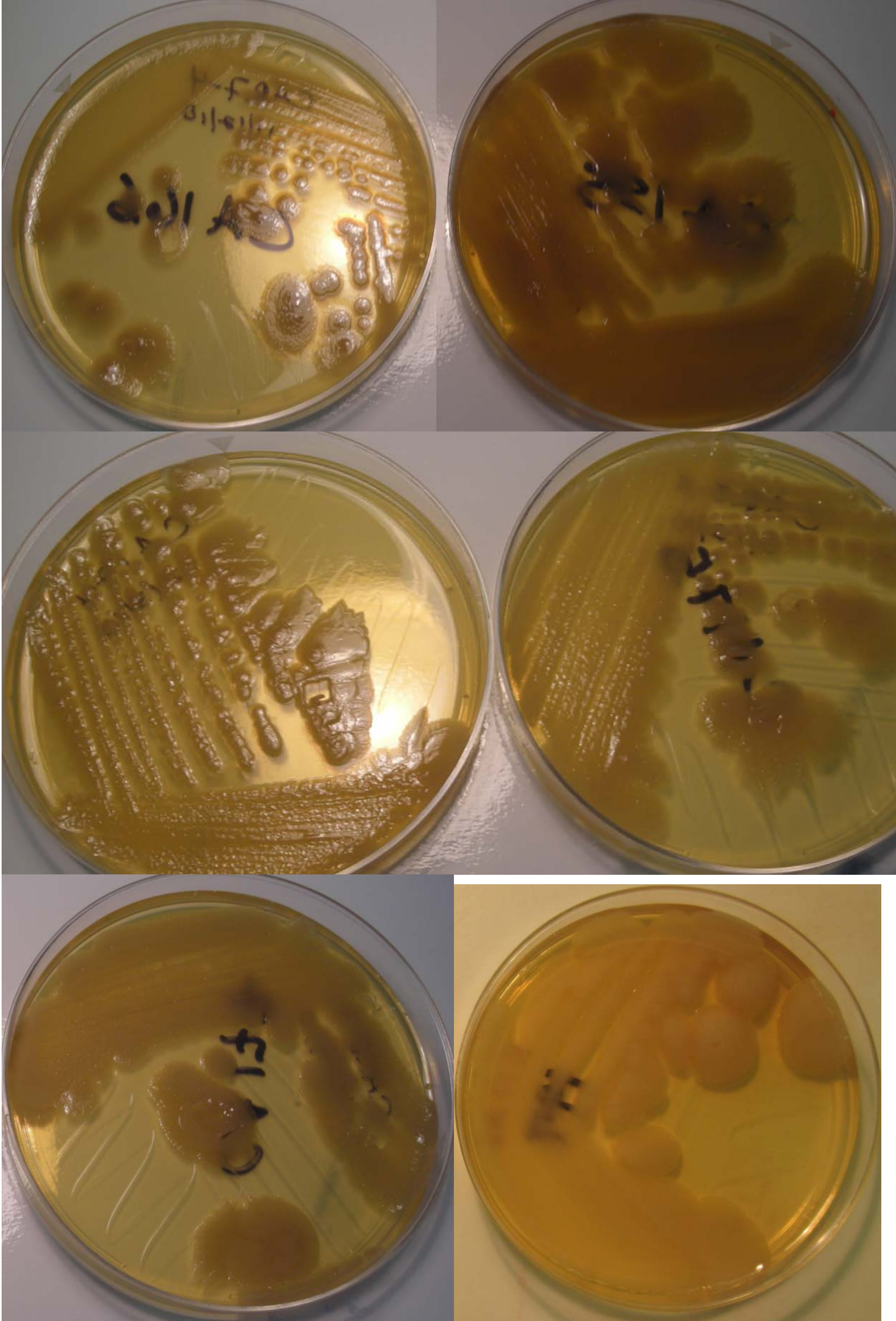
Bacillus megaterium



Bacillus cereus group



Bacillus simplex



Production of *Bacillus* Inoculum

Anna Testen

Overview

- Isolating Bacilli
- Producing Inoculum
- Testing Inoculum

Isolating Bacilli

- Bacilli form heat tolerant endospores
- Use heat to kill other bacteria, but Bacilli will remain

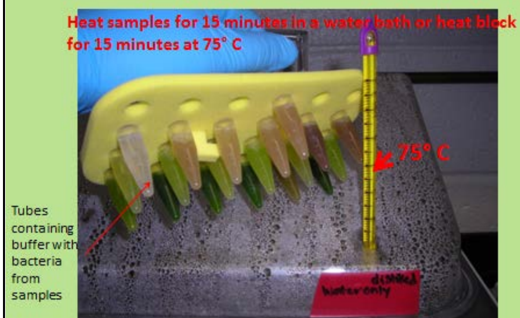
Isolating Bacilli Step 1: Surface Disinfest Tissue

Add sample to sterile bag and crush with hammer or mortar and pestle

Collect bacteria in sample

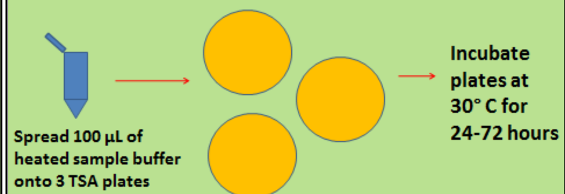
1. Add 3-5 mL of 0.1 M sterile phosphate buffer to sample
2. Mix thoroughly for 1 minute
3. Remove buffer that now contains bacteria from the sample and place in sterile tubes

Step :Heat samples to kill other bacteria



Tubes containing buffer with bacteria from samples

Plate heated samples onto nutrient media



Select well isolated colonies and streak onto TSA to obtain a pure culture

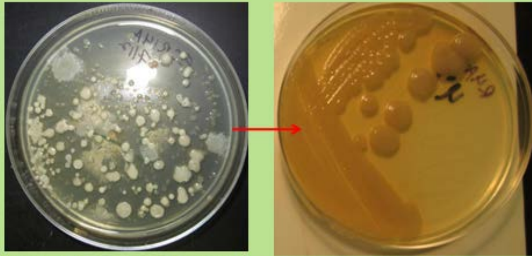
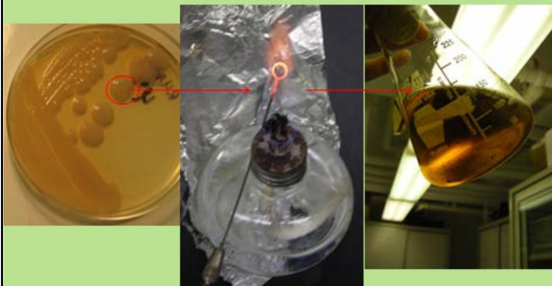


Plate after 72 hours incubation **Pure culture obtained from subculturing colony on first plate**

Media for Inoculum Production

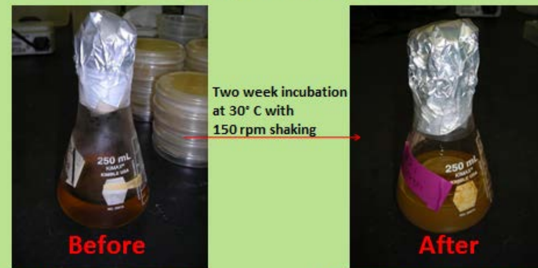
- Tryptic Soy Agar (TSA)
  - 20 g peptone (17 g from casein peptone, 3 from soy peptone)
  - 2.5 g glucose
  - 5 g NaCl
  - 2.5 g K_2HPO_4
  - 15 g agar
- Nutrient Agar
  - 5 g peptone
  - 3 g beef extract
  - 5 g NaCl
  - 15 g agar

Inoculating the Media



Pick a 24 hour old colony with a sterile loop and inoculate into sterile nutrient broth

Growing the Cultures



Seal cultures with a foam or cotton plug, cover with aluminum foil and seal with parafilm

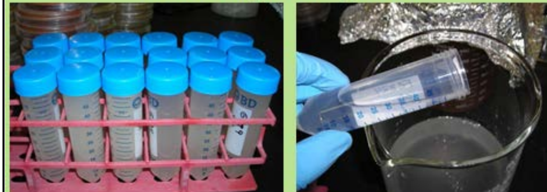
After two weeks, the media will be turbid and the bacillus should have exhausted the nutrients and formed endospores

Harvesting the Spores

Pour mature cultures into sterile tubes

Centrifuge tubes for 15 minutes at ~5,000 rpm to pellet the endospores. Pour off the supernatant.

Cleaning the Spores

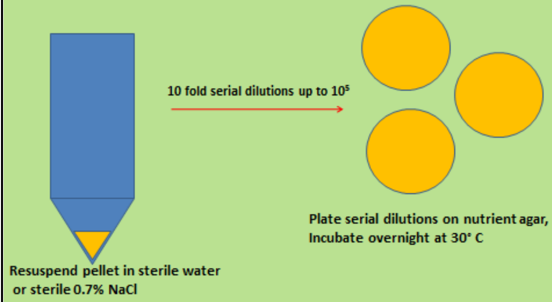


Resuspend pellet in sterile water by shaking or vortexing. Centrifuge to pellet Bacilli.

Pour off the water supernatant.

Repeat 1-2 times more to remove all germination inhibitors and other bacterial waste products.

Measuring Spore Concentration



To determine concentration of spores in original tube

$$\frac{\text{Avg. \# of colonies on plate}}{\text{0.1 mL plated}} \times \frac{1}{\text{Dilution Factor}} = \text{\# of original colony forming units per mL}$$

Example: If 51, 46, and 60 colonies were counted on plates that were plated using 100 µL of a 10^{-5} dilution of the original spore suspension, there would be 5.23×10^7 bacteria per mL

Media Recipes

- 0.7% Saline
  - 7 g NaCl in 1 L water
- 0.1 M Phosphate buffer (1 L)
  - 13.6 g potassium phosphate monobasic
  - 17.4 g potassium phosphate dibasic

Protocol for Screening Microbes for the Ability to Produce Chitinase

Preparation of Hydrolyzed Chitin (adapted from (74))

1. Stir 600 mL of concentrated hydrochloric acid (HCl) into 60 g of chitin in a large (> 4 L) beaker or bucket. Stir this mixture 3-4 times during a 30 minute period.
2. After the mixture has settled for 30 minutes, add 4 liters of water and stir. Let the mixture sit overnight.
3. In the morning, pour off the liquid, and add another 4 liters of water. Let this settle and again pour off the water. Repeat this process until the pH of the water is greater than 2.
4. Drain off the remaining liquid and stir the remaining mixture well. Take out 10 mL of the mixture and dry in a tared weighboat to determine the percent of chitin per mL of mixture. Adjust the mixture to 1% chitin.

Chitin Nutrient Agar (500 mL)

1. Blend 200 mL of 1% hydrolyzed chitin with 300 mL of 0.1 M phosphate buffer in a blender
2. Add 2 g of nutrient agar and 10-15 g of agar
3. Make sure the pH of the media is above 6
4. Autoclave and pour plates

Testing Bacterial Isolates for Chitinase Production

1. Divide plate into quadrants and stab isolates in triplicate into the chitin nutrient agar.
2. Incubate plates in the dark at 30° C for 7-10 days.
3. Look for clearing halos after incubation. If halos are produced, the isolate can produce chitinase.

Production of Rifampicin Resistant Bacterial Mutants (21; 62; 133)

Materials Needed

-TSA with 100 µg/mL rifampicin (make a stock of 50 mg/mL of rifampicin in methanol or DMSO)

-TSA with 50 µg/mL rifampicin

-50 mL of TSB in Erlenmeyer flasks

1. Grow isolates in TSB for 48 hours. Spin down cultures, discard supernatant and resuspend bacteria in 5 mL of sterile water
2. Spread100 µL of bacteria onto TSA with 100 µg/mL rifampicin. Incubate plates in the dark for 3-5 days at 30° C.
3. Select colonies that appear to be resistant and still have normal morphology. Passage these sub-isolates on TSA with 50 µg/mL rifampicin 5 times.
4. Passage resistant isolates on TSA without antibiotic 5-10 times and finally culture them on TSA with 50 µg/mL rifampicin to ensure that the mutation is stable.
5. Grow isolates in TSB with 50 µg/mL to produce endospores as usual. Inoculate onto plants or seeds, as desired. To reisolate resistant mutants, plate plant samples on TSA with 50 µg/mL rifampicin.

Measuring available phosphate in liquid NBRIP

-Erlenmeyer flasks with 50-100 mL of liquid NBRIP

-Reagents for Murphy and Riley phosphate assay (91)

-5 N sulfuric acid

-ammonium molybdate (20 g ammonium molybdate, bring to 500 mL in distilled water)

-ascorbic acid (1.32 g ascorbic acid in 75 mL water, make fresh every time)

-potassium antimonyl tartrate (0.2743 g potassium antimoynl tartrate, bring to 100 mL in distilled water)
-phosphate standard (0.1757 g KH_2PO_4 in 1 liter distilled water, this equals 40 µg/mL phosphate)

1. Inoculate liquid NBRIP with bacterial isolate to be studied. Incubate at 30° C with shaking for 1 week (or desired amount of time).

2. Remove inoculated NBRIP and place in centrifuge tubes. Spin at 5k rpm for 3-5 minutes to pellet bacteria and remaining tricalcium phosphate.

3. Remove 40 mL of cleared supernatant and place in fresh tube. To 40 mL of cleared supernatant add 8 mL of Murphy Riley mixed reagent (mix 125 mL 5 N sulfuric acid, 37.5 mL ammonium molybdate, 75 mL ascorbic acid and 12.5 mL potassium antimoyl tartrate) and 2 mL of distilled water. Mix well and let sit for 10 minutes.

4. Measure absorbance at 880 and compare to standard curve to determine the amount of available phosphate.

Appendix A

First Report of Quinoa Downy Mildew Caused by *Peronospora variabilis* in the United States

A. L. Testen, Dept. of Plant Pathology, The Pennsylvania State University, University Park, 16802; J. M. McKemy, USDA-APHIS-PPQ-National Identification Services, Beltsville, 20705; and P. A. Backman, Dept. of Plant Pathology, The Pennsylvania State University, University Park, 16802.

Quinoa, *Chenopodium quinoa* Willd., is an Andean crop prized for high nutritional value and adaptability to harsh environments. Quinoa is plagued by downy mildew caused by *Peronospora variabilis* Gäum (formerly *Peronospora farinosa* f. sp. *chenopodii* Byford) (2). Quinoa production has spread beyond native Andean ranges and quinoa downy mildew has been reported in India, Canada, and Denmark (2). During summer 2011, quinoa trials were established to determine the ability of quinoa to grow under Mid-Atlantic conditions and monitor for regional disease problems. In July, after cool, rainy conditions, downy mildew-like symptoms were observed on quinoa at research plots in Centre Co. and Lancaster Co., PA. Symptoms and signs consisted of irregularly shaped areas of foliar chlorosis or pink discoloration accompanied by dense, gray sporulation on both leaf surfaces. Sporangia were tan to gray-brown, semi-ovoid, often with a pedicel, mean length of 31 μm, and mean width of 23 μm. Sporangiophores branched dichotomously, and the terminal branchlets curved and tapered to a point. Orange oospores were present in field samples of leaf tissue. DNA was extracted from infected foliar tissue and sporangial suspensions. A semi-nested PCR protocol (3) was used to obtain partial ITS sequences of six *Peronospora* isolates. The sequences shared 99% maximum identity to a known *P. variabilis* accession (FM863721.2) in GenBank. A voucher specimen was deposited into the U.S. National Fungus Collections (BPI 882064). Pathogenicity of each of two strains of *P. variabilis* was confirmed by inoculating quinoa using sporangia (4). Sporangia were shaken from leaves in sterile distilled water, and the suspension was filtered through cheesecloth. A

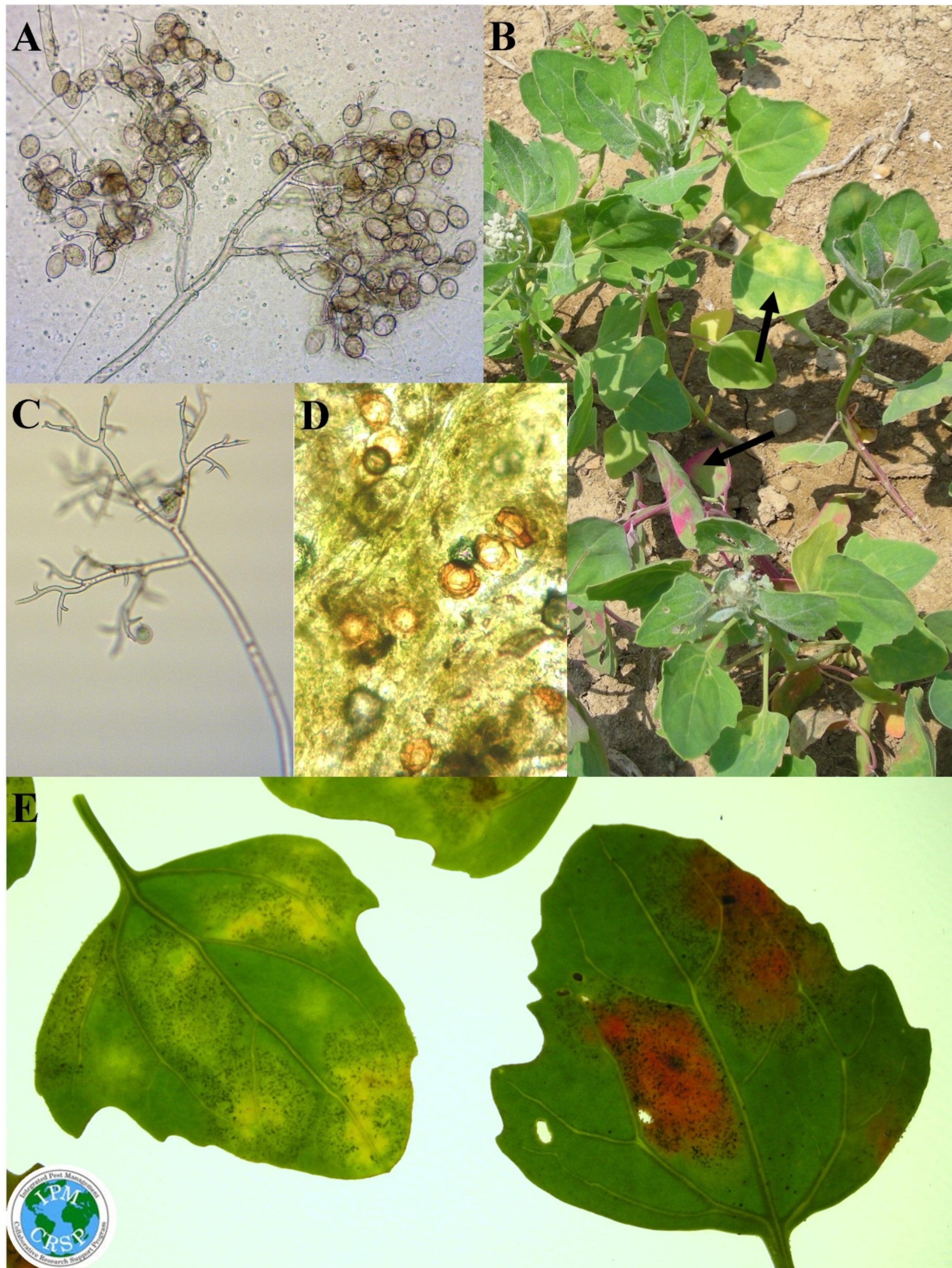
0.01% Tween solution was added and the suspension diluted to 10^3 sporangia/ml. Using an atomizer, 10 ml sporangial suspension (or sterile water for noninoculated control plants) was sprayed onto one flat of 18 two-week old quinoa plants, and relative humidity was increased to saturation using a humidity dome for 24 h. After one week, chlorosis and pink discoloration were noted on leaves of inoculated quinoa, and after 18 h of subsequent increased humidity (>95% RH), dense gray sporulation was observed. No symptoms were noted on non-inoculated control plants. Sporangia and sporangiophores were examined morphologically and confirmed to be *P. variabilis*, confirming Koch's postulates. For culture maintenance, two week old quinoa leaves were placed onto a sporangial suspension on top of 1% water agar and maintained in a growth chamber at 20°C with 16 h light/day. Quinoa downy mildew is seedborne (1) and initial infections may have occurred from oospores in the pericarp, despite intensive processing of consumable quinoa seeds to remove saponins. To our knowledge, this is the first report of quinoa downy mildew in the United States and is also the first report of *P. variabilis* in the United States.

References: 1) S. Danielson et al. Seed Sci. Tech. 32:91, 2004. 2) Y. Choi et al. Mycopath. 169:403, 2010. 3) D. Cooke et al. Fun. Gen. Bio. 30:17, 2000. 4) J. Ochoa et al. Plant Path. 48:425, 1999.

Figure A Signs and symptoms of quinoa downy mildew

A) Gross morphology of sporangiophore and sporangia of *Peronospora variabilis*. B) Field symptoms of quinoa downy mildew caused by *P. variabilis* (arrows point to typical yellow and pink foliar discoloration). C) Sporangiophore branching pattern of *P. variabilis*. D) Oospores of *P. variabilis* in quinoa (*Chenopodium quinoa*) leaf tissue. E) Characteristic yellow and pink

foliar discoloration accompanied by dense gray sporulation of *P. variabilis* on quinoa.



Appendix B

First Report of *Passalora* Leaf Spot of Quinoa Caused by *Passalora dubia* in the United States

A. L. Testen, Dept. of Plant Pathology, The Pennsylvania State University, University Park, PA 16802; J. M. McKemy, USDA-APHIS-PPQ-National Identification Services, Beltsville, MD 20705; and P. A. Backman, Dept. of Plant Pathology, The Pennsylvania State University, University Park, PA 16802.

The Andean seed crop quinoa, *Chenopodium quinoa* Willd., is an important export of Bolivia, Ecuador, and Peru. Key foliar diseases of quinoa include quinoa downy mildew (caused by *Peronospora variabilis* Gäum; 1), *Ascochyta* leaf spot (caused by *Ascochyta* sp.; 1), and a *Cercospora*-like leaf spot, the latter of which has been observed on cultivated quinoa (Jose B. Ochoa, *unpublished*) and native *Chenopodium* species. *Passalora dubia* (Riess) U. Braun (syn. *Cercospora dubia*) was tested in Europe as a biological control agent for *Chenopodium album* (2) and has been reported on *C. album* in the United States (U.S. National Fungus Collections). Quinoa field plots were established in Pennsylvania during summer 2011 and *Cercospora*-like leaf spot symptoms were first observed on quinoa in Centre Co. and Lancaster Co. in August 2011, after an extended rainy period. Foliar symptoms were round to oval, brown to grey-black lesions, less than 1 cm in diameter, with darker brown, reddish margins. Similar symptoms were observed on *C. album* weeds within both fields. Using a hand-lens, conidia were observed within sporulating lesions. Conidia were hyaline and septate, 25 to 98 µm × 5 to 10 µm, and had an average of 6 cells per conidium. The fungus was isolated by picking single conidia from sporulating lesions (under a dissecting scope) and incubated on V8 agar in the dark at 20°C to induce sporulation. For DNA extraction, cultures were grown in potato dextrose broth amended with yeast extract. The internal transcribed spacer (ITS) region was amplified using primers ITS4 and ITS5 (3), and the resulting sequence shared 99% maximum identity with a vouchered isolate

of *P. dubia* (GenBank EF535655). To test the pathogenicity of our *P. dubia* isolate, 5.9 x 103 conidia per mL (suspended in sterile water with 0.1% Tween 20) or the control solution with no conidia were sprayed, using an atomizer, onto two month-old quinoa plants, with 18 replications per treatment. Plants were covered with a humidity dome and maintained at > 99% RH for 48 h. Plants were grown in the greenhouse at approximately 65% RH. After one month, circular to oval, light brown lesions (< 1 cm diameter) with darker margins were observed on approximately 10% of the leaves of inoculated plants, whereas no symptoms were observed on the control plants. Infected leaves were collected, incubated in a humidity chamber, and conidia were picked from sporulating lesions and inoculated onto V8 agar amended with 3% (w/v) fresh, ground quinoa plant tissue (4). Cultures were maintained at 20° C with 16 hour photoperiod to induce sporulation. The identity of the re-isolated fungus was confirmed morphologically and by DNA sequencing to be identical to the isolate used to test Koch's Postulates. *P. dubia* was also isolated from *C. album* lesions and infected *C. album* may have served as a source of inoculum for quinoa. This is the first report of *Passalora* leaf spot of quinoa in the United States.

References: 1) S. Danielsen Food Rev. Int. 19: 43, 2003. 2) P. Scheepens et al. Integ. Pest. Man. Rev. 2: 71, 1997. 3) S. Goodwin et al. Phytopath. 91: 648, 2001. 4) M. Vathakos Phytopath. 69: 832, 1979.

Figure B Signs and Symptoms of *Passalora* Leaf Spot of Quinoa

A) Typical foliar symptoms of *Passalora* leaf spot of quinoa (*Chenopodium quinoa*) caused by *Passalora dubia* B) Conidia of *Passalora dubia* isolated from quinoa

Appendix C

First Report of Ascochyta Leaf Spot of Quinoa Caused by *Ascochyta sp.* in the United States

A. L. Testen, Dept. of Plant Pathology, The Pennsylvania State University, University Park, 16802; J. M. McKemy, USDA-APHIS-PPQ-National Identification Services, Beltsville, MD 20705; and P. A. Backman, Dept. of Plant Pathology, The Pennsylvania State University, University Park, 16802.

Quinoa (*Chenopodium quinoa* Willd.), an Amaranthaceous pseudo-grain, is native to the Andean region of South America and is an important food and export crop for this region. Quinoa is susceptible to several foliar diseases including *Ascochyta* leaf spot reportedly caused by *Ascochyta hyalospora* or *Ascochyta caulina* (1, 2). *A. hyalospora* can be transmitted on quinoa seeds (3) and *A. caulina* has been studied as a biological control agent of other *Chenopodium* species, such as *Chenopodium album* (4). Field trials of quinoa were established in Pennsylvania during summer 2011. Widespread fungal-like leafspot symptoms were first observed on quinoa plants in mid-August 2011 in the quinoa planting in Centre County, PA. Tan to reddish-brown, irregularly shaped lesions were observed with numerous black pycnidia within each lesion. Crushing the pycnidia revealed subhyaline to light brown, 1-2 –(3) septate, cylindrical to ovoid spores, with length of 13-25 µm by width of 5-10 µm, characteristic of *Ascochyta* conidia. Cultures of *Ascochyta* were obtained by plating pycnidia from surface sterilized leaves onto ½ strength acidified potato dextrose agar (APDA). DNA was extracted from cultures of *Ascochyta* and amplified using ITS4 and ITS5 primers. Sequences obtained from the amplified DNA shared 99% maximum identity with a GenBank accession of *Ascochyta obiones* (GU230752.1). To obtain conidia for pathogenicity trials, cultures were transferred to oatmeal agar and placed in a 20° C incubator with a 12 hour photoperiod. Conidia were harvested by scraping cultures after 2 weeks of growth. The conidial suspension was filtered through cheesecloth and adjusted to 1.8 x 10^5 conidia/mL. 0.1% Tween 20 was added to the final

inoculum and sprayed (using a Crown Spra-tool) onto 10 one-month old quinoa plants. For negative controls, 6 plants were sprayed with sterile water with 0.1% Tween 20. Plants were placed into a growth chamber and were bagged for 48 hours to maintain >95% humidity. After 48 hours, tan irregularly shaped lesions were observed on inoculated plants, but no symptoms were observed on non-inoculated control plants. The plants were allowed to grow for two more weeks to observe symptom development, after which time leaves with characteristic lesions were collected for isolation. Symptomatic leaves were surface sterilized in 10% bleach for 1 minute and tissue from the periphery of the lesions was plated onto ½ APDA. Cultures of *Ascochyta* sp. were reisolated and confirmed by morphology and sequencing of the ITS region. The obtained pathogen is morphologically similar to either *A. hyalospora* or *Ascochyta chenopodii* (not *A. caulina*). At this time, a lack of ITS sequence data for these two species in GenBank prevents a final identification to species. To our knowledge, this is the first report of *Ascochyta* leaf spot of quinoa in the United States.

References: 1) S. Danielsen Food Rev. Int. 19: 43, 2003. 2) M. Drimalkova. Plant Protect. Sci. 39: 146, 2003. 3) G. Boerema. Neth. J. Plant. Path. 83: 153, 1977.4) P. Scheepens et al. Integ. Pest. Man. Rev. 2: 71,1997.

Figure C Signs and Symptoms of *Ascochyta* Leaf Spot of Quinoa

A) Typical foliar symptoms of *Ascochyta* leaf spot of quinoa B) Pycnidiospores of *Ascochyta sp.*

Appendix D

Plant Growth Promoting Characteristics of Bolivian *Bacillus* and *Paenibacillus* Isolates

Bolivian isolates of *Bacillus* and *Paenibacillus* were screened for the ability to solubilize phosphate, produce chitinase and produce indole acetic acid (IAA). Details on the acquisition of this population of isolates (BV) can be found in chapter 2 along with methods for screening for the tricalcium phosphate solubilizing phenotype. Methods for screening for IAA and chitinase production can be found in chapter 5.

Isolate	Species group or Species	Tricalcium Phosphate Solubilizing	Chitinase Production	IAA Production
BV5	*B. cereus* group	X	X	
BV31	*Paenibacillus*	X	X	
BV104	*Paenibacillus*	X	X	
BV127	*B. cereus* group	X	X	
BV154	*B. simplex*	X	X	
BV229	*B. pumilus*	X	X	
BV231	*B. pumilus*	X	X	
BV232	*B. pumilus*	X	X	
BV265	*B. circulans*	X	X	
BV326	*B. pumilus*	X	X	
BV327	*B. pumilus*	X	X	
BV342	*B. pumilus*	X	X	
BV6	*B. simplex*	X		X
BV13	*Paenibacillus*	X		X
BV15	*B. simplex*	X		X
BV19	*B. simplex*	X		X
BV20	*B. simplex*	X		X
BV23	*Paenibacillus*	X		X
BV24	*B. simplex*	X		X
BV27	*B. simplex*	X		X
BV34	*B. simplex*	X		X
BV45	*B. simplex*	X		X
BV55	*B. simplex*	X		X
BV57	*B. simplex*	X		X
BV64	*B. simplex*	X		X
BV65	*B. simplex*	X		X
BV67	*B. simplex*	X		X
BV69	*B. simplex*	X		X

Isolate	Species group or Species	Tricalcium Phosphate Solubilizing	Chitinase Production	IAA Production
BV70	*B. simplex*	X		X
BV71	*Paenibacillus*	X		X
BV77	*B. simplex*	X		X
BV85	*B. simplex*	X		X
BV88	*B. simplex*	X		X
BV95	*Paenibacillus*	X		X
BV98	*B. simplex*	X		X
BV108	*Paenibacillus*	X		X
BV122	*Paenibacillus*	X		X
BV123	*B. simplex*	X		X
BV128	*B. simplex*	X		X
BV130	*B. simplex*	X		X
BV132	*Paenibacillus*	X		X
BV135	*B. simplex*	X		X
BV142	*Paenibacillus*	X		X
BV144	*B. megaterium*	X		X
BV145	*B. simplex*	X		X
BV155	*B. simplex*	X		X
BV159	*B. simplex*	X		X
BV170	*B. simplex*	X		X
BV193	*B. simplex*	X		X
BV202	*B. simplex*	X		X
BV204	*B. babaricus*	X		X
BV207	*B. simplex*	X		X
BV221	*B. simplex*	X		X
BV237	*B. simplex*	X		X
BV241	*B. simplex*	X		X
BV252	*B. simplex*	X		X
BV268	*B. circulans*	X		X
BV306	*Paenibacillus*	X		X
BV312	*Paenibacillus*	X		X
BV316	-	X		X
BV330	*Paenibacillus*	X		X
BV333	*Paenibacillus*	X		X
BV102	*Paenibacillus*		X	X
BV249	*Paenibacillus*		X	X
BV254	*Paenibacillus*		X	X
BV256	*Paenibacillus*		X	X
BV260	*Paenibacillus*		X	X
BV261	*Paenibacillus*		X	X
BV262	*Paenibacillus*		X	X
BV8	*Paenibacillus*	X	X	X

Isolate	Species group or Species	Tricalcium Phosphate Solubilizing	Chitinase Production	IAA Production
BV10	*Paenibacillus*	X	X	X
BV16	*Paenibacillus*	X	X	X
BV25	*Paenibacillus*	X	X	X
BV36	*Paenibacillus*	X	X	X
BV40	*Paenibacillus*	X	X	X
BV41	*Paenibacillus*	X	X	X
BV42	*Paenibacillus*	X	X	X
BV48	*Paenibacillus*	X	X	X
BV50	*Paenibacillus*	X	X	X
BV52	*Paenibacillus*	X	X	X
BV59	*Paenibacillus*	X	X	X
BV61	*Paenibacillus*	X	X	X
BV63	*Paenibacillus*	X	X	X
BV72	*Paenibacillus*	X	X	X
BV74	*Paenibacillus*	X	X	X
BV76	*Paenibacillus*	X	X	X
BV92	*Paenibacillus*	X	X	X
BV97	*Paenibacillus*	X	X	X
BV103	*Paenibacillus*	X	X	X
BV138	*Paenibacillus*	X	X	X
BV172	*Paenibacillus*	X	X	X
BV186	*Paenibacillus*	X	X	X
BV201	*Paenibacillus*	X	X	X
BV209	*Paenibacillus*	X	X	X
BV214	*Paenibacillus*	X	X	X
BV222	*Paenibacillus*	X	X	X
BV224	*Paenibacillus*	X	X	X
BV228	*Paenibacillus*	X	X	X
BV235	*Paenibacillus*	X	X	X
BV239	*Paenibacillus*	X	X	X
BV243	*Paenibacillus*	X	X	X
BV245	*B. simplex*	X	X	X
BV289	*Paenibacillus*	X	X	X
BV298	*Paenibacillus*	X	X	X

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