Beneficial Fungal Interactions Resulting in Accelerated Germination of *Astragalus utahensis*, a Hard-Seeded Legume

by

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GRADUATE COMMITTEE APPROVAL

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

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ABSTRACT

Beneficial Fungal Interactions Resulting in Accelerated Germination

of *Astragalus utahensis*, a Hard-Seeded Legume

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Seed germination is pivotal in the life cycle of native plants in a restorative context because initiation of the metabolic processes critical to establishment is key to survival in such a competitive environment. Dormancy characteristics of some native plants including the subject species, *Astragalus utahensis,* have evolved mechanisms to control germination in order to maintain a seed bank and ensure germination at the right time under optimal conditions. *In vitro* germination studies confirm beneficial interactions between *Alternaria* and *Aspergillus* fungi and *Astragalus utahensis* seed. Inoculated seed trials (1.0×10^6 spores/mL) exhibited a highly significant difference in percent germination between the uninoculated control at 5.0 % germination and seeds inoculated with *Alternaria* and *Aspergillus* germinating at 95 % and 55 %, respectively. Germination trials conducted in the greenhouse revealed a beneficial relationship

between fungal spore inoculation and seed germination. Control seeds germinated in soil at a rate of 16.0 %; three times as high as exhibited *in vitro*. Seed inoculated with either *Alternaria* or *Aspergillus* seeds germinated in soil at the same rate of 50.0 %. A seed germination trial conducted in the field demonstrated a beneficial response with *Aspergillus* inoculation. Fall plantings on two sites near Fountain Green and Nephi, Utah confirm this beneficial response to *Aspergillus* spore inoculation. These field trials indicated a highly significant response with the germination of scarified control seed at 14.7 % and the *Aspergillus* and *Alternaria* treated seed germinating at 29.3 and 19.3 %, respectively. Greenhouse germination trials with spore-inoculated seed indicated a 100% survival rate. *Astragalus utahensis* seeds germinated at an accelerated rate when inoculated with *Aspergillus* and *Alternaria* spores in-vitro. The beneficial germination response of fungal inoculated seeds indicates the efficacy of these treatments in dormancy contravention in hard-seeded species.

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Beneficial Fungal Interactions Resulting in Accelerated Germination of *Astragalus utahensis*, a Hard-Seeded Legume

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Abstract

Seed germination is pivotal in the life cycle of native plants in a restorative context because initiation of the metabolic processes critical to establishment is key to survival in such a competitive environment. Dormancy characteristics of some native plants including the subject species, *Astragalus utahensis*, have evolved mechanisms to control germination in order to maintain a seed bank and ensure germination at the right time under optimal conditions. In-vitro germination studies confirm beneficial interactions between *Alternaria* and *Aspergillus* fungi and *Astragalus utahensis* seed. Inoculated seed trials (1.0×10^6 spores/mL) exhibited a highly significant difference in percent germination between the uninoculated control at 5.0 % germination and seeds inoculated with *Alternaria* and *Aspergillus* germinating at 95 % and 55 %, respectively. Germination trials conducted in the greenhouse revealed a beneficial relationship between fungal spore inoculation and seed germination. Control seeds germinated in soil at a rate of 16.0 %; three times as high as exhibited in-vitro. Seed inoculated with either *Alternaria* or *Aspergillus* seeds germinated in soil at the same rate of 50.0 %. A seed germination trial conducted in the field demonstrated a beneficial response with *Aspergillus* inoculation. Fall plantings on two sites near Fountain Green and Nephi, Utah confirm this benefical response to *Aspergillus* spore inoculation. These field trials indicated a highly significant response with the germination of scarified control seed at 14.7 % and the *Aspergillus* and *Alternaria* treated seed germinating at 29.3 and 19.3 %, respectively. Greenhouse germination trials with spore inoculated seed indicated a 100% survival rate. *Astragalus utahensis* seeds germinated at an accelerated rate when inoculated with *Aspergillus* and *Alternaria* spores in-vitro. The beneficial germination

response of fungal inoculated seeds indicates the efficacy of these treatments in dormancy contravention in hard-seeded species.

Introduction

Seed germination (Vasquez-Ramos et al., 2003) is the initiation of processes involved in metabolism and development of the dormant seed. Germination is pivotal in the life cycle of a plant, without it and in the absence of other reproductive mechanisms life would not ensue (Stiles, 1948). The quicker dormancy is circumvented and germination occurs the speedier the establishment characteristics of the plant can take over and work to ensure survivability.

Evenari (1962) identifies seed germination in a xeric environment as critical to the life cycle of xerophytes because the speed, timing, and other intricacies of germination determine its survival. An outline (Evenari, 1962) of optimal germination and establishment conditions for species in an arid environment reports that germination should only take place at a time when moisture conditions are optimal, germination should be prompt, not all seeds or dispersal units produced in any one year should germinate simultaneously leaving a reserve of viable dispersal units in the soil and germination should be restricted to habitats that offer the greatest chance for survival. Under natural conditions of moderate competition and less than extreme circumstances these optimum conditions can be substantially met by the physiology and life history of most hard-seeded arid plants including *Astragalus utahensis.*

Dormancy is a complex interaction between external environmental and inherent internal germination barriers that must be breached if germination is to occur (Baskin and Baskin, 1998). Green (1973) suggests that, in a natural setting, seed dormancy is

ecologically significant because it delays germination until conditions are right for the establishment of seedlings. This argument is strengthened when he suggests that dormancy is critical to the lifecycle of plants living in environmentally unstable conditions because it allows for the maintenance of an annual seed bank. This germ bank allows for relatively keen flexibility in terms of rapid germ response in ideal conditions and continued viability during perilous seasons. In short dormancy or control of seed germination is critical to the survival of species under certain, undesirable, environmental conditions.

Competition between plant species for light, water, oxygen, carbon dioxide, and nutrients (Caudle, 1968) creates a dynamic demand for these growth variables. Donald (1963) describes competition as a condition that does not occur unless there is a shortage of one factor that is immediately necessary for the survival of more than one species in an ecosystem. An abundant supply of these materials in a post-fire ecosystem would seem like an ideally non-competitive environment. This profusion of growth factors, however, sets the vital requirement of early establishment for native species. Although *Astragalus utahensis* is able to exist in recently disturbed areas under relatively harsh environmental conditions having excellent survival characteristics in terms of rapid growth and completion of its life cycle, it is unable to compete well with invasive plants (Green, 1973) thus leaving it at a disadvantage. Life cycles of invasive species, on the other hand, have developed rapid, uniform germination characteristics that are integral to rapid establishment in harsh environments. The critical point in competition for resources arrives quickly creating a hostile environment for native plants unable to acquire sufficient resources for establishment and survival. Successfully competing for

factors necessary to plant establishment and survival is a function of the plants ability to grow rapidly and complete its life cycle before other plants are able to commandeer these same resources for similar purposes (Caudle, 1968).

Bibbey (1948) points to the quick germ response of weed seeds as the characteristic, independent of the species competitive abilities and seed production, which allows them to maintain viability in the face of disturbances. This faculty of invasive species frequently hampers revegetation, via native species, in post-disturbance ecosystems and thus constitutes a formidable barrier to restoration; prompt germination of native species in such a competitive domain is crucial to their establishment and survival. The inability of some native species to promptly establish metabolic seed activity, even under optimal conditions, constitutes a formidable barrier to their restorative capacity.

The Bureau of Land Management in conjunction with the Forest service has been working to return stability to the Great Basin where in 1999 extensive wildfires burned about 1.7 million acres. Ainitiative, was developed to manage restoration efforts and coordinate organizations involved. Managers and scientists recognized the need to supplement established wildland restoration methods derived from small-scale management efforts in order to restore balance to the system before invasive species monopolized resources and cycles inherent to the survival of endemic species. The Great Basin Native Plant Selection and Increase Project was developed as part of the original initiative to research native plant species with the aim of selecting candidates suitable for restoration in terms of their life history and competitive abilities and working to improve production capabilities for these species.

Investigation into the viability of the use of hard seeded species in restoration requires specific attention be given to the nature of their seed dormancy as described. Researchers have made it abundantly clear that germination of the genus *Astragalus* is a function of multiple factors (Ziemkiewicz and Cronin, 1981) including: temperature, moisture, water potential, seed source, presence of leachable inhibitory chemicals, and seed hardness together with integrity (Baskin and Quarterman, 1969). These dormancy mechanisms stand as prime, inhibitory factors in this hard seeded species germination. Without physical or chemical triggering mechanisms seeds could remain dormant for several years (Baskin and Quarterman, 1969) and while the evolutionary advantages are great in this context, the restorative advantages are nominal. In the framework of conditions outlined by Evenari (1962) germ response in a restorative scenario should occur quickly. However, in contrast to Evenari's discussion (1962) it would be beneficial for hard seed to germinate homogeneously, in order to retain some ecological competitiveness with species exhibiting more invasive characteristics.

Two fungi were isolated from *Astragalus utahensis* for use as inoculum during the course of this study; each has a ubiquitous distribution (Rotem, 1994). These fungi, *Alternaria* and *Aspergillus*, belong to the Deuteromycetes (imperfect fungi) (Agrios, 2005) rely solely on asexual conidia for reproduction. Both fungi are adaptive to fit any ecological ranging from detritus to the phyloplane (Spooner and Roberts, 2005). Although these fungi belong to the same family they differ in morphology, pathology, and growth requirements.

Alternaria is described by Rotem (1994) as either parasitic or saprophytic and thus many factors influence the growth and pathogenicity of this fungus including

moisture regime, nutrient availability, and temperature (Agarwal and Sinclair, 1997). Rotem (1994) addresses these factors by indicating that a moisture saturated environment is ideal for spore germination and production, the presence of leachate or other seed exudates accelerates spore germination at the optimal temperature of 25 °C. Wounding and similar conditions provide the optimal situations for infection by this weak parasite and thus can be linked to the pathogenicity of this fungus (Rotem, 1994).

Alternatively, *Aspergillus* is described as a saprophyte (Cotty et al., 1994) though it is associated with a variety of plant diseases. The growth of this fungus can be influenced by a number of environmental factors including moisture availability, temperature and other factors like gas composition and pH (Kozakiewicz and Smith, 1994). *Aspergillus* is mesophillic with optimal growth occurring at temperatures between 10 and 40 °C (Kozakiewicz and Smith, 1994) thriving in dry and droughty conditions where lack of moisture is common (Cotty et al., 1994).

The objectives of this study are twofold. First, establish the in-vitro germination response relationship manifest when seeds of *Astragalus utahensis* are inoculated with spores of the two test fungi originally isolated from *Astragalus utahensis* germination trials. Second, demonstrate the viability of this response outside of the Petri dish by testing the accelerated response in increasingly variable and complex soil environments such as the growth chamber, greenhouse and field.

Materials and Methods

Astragalus utahensis, Utah milkvetch, seed was selected from lot U5-02 to study germination response when inoculated with Aspergillus and Alternaria fungi. Lot U5-02 was collected June 2002 from a site, elevation 1483 m, located on the outskirts of Spanish Fork, Utah at the northeast corner of the intersection of US 89 and US 6 (UTM Zone 12S, E. 449964 N. 4437199). This seed was randomly selected from a relatively uniform population of *A. utahensis* growing on an old river delta in shallow soil.

Fungal cultures of *Aspergillus* and *Alternaria* were isolated from *A. utahensis* seed and used for the production of inoculum to test their influence on germination. *Alternaria* and *Aspergillus,* both species are unknown, were grown and maintained on weak nutrient solution of 1/3 strength potato dextrose agar (1/3 PDA).

The hard-seeded nature of *A. utahensis* required that it be acid scarified (98.0 % H_2SO_4) for up to 20 minutes in order to facilitate imbibition and eventual germination. Scarification was performed in lots of 80 to 150 seeds with up to 8 lots scarified at a time. Placement of seeds in cone sieves, created from polypropylene mesh, facilitated uniform exposure to acid and rapid removal of seed post scarification. After seeds were removed from acid treatment they were immediately rinsed with ddH_2O for 10 seconds in order to neutralize exothermic reactions taking place. A pressurized rinse with distilled water (25 to 30 seconds) completely neutralized the acid ensuring that the seed and its container were clean. Seed was maintained in aseptic conditions in a laminar flow hood until plated or stored to prevent contamination by an outside source.

After acid scarification, the seeds were inoculated with a spore solution created from sporulating cultures approximately 1 to 2 months month old. The fungal inoculum

was created by adding reverse osmosis water to the petri culture and gently scraping the mycelium to dislodge the spores. One drop of Tween 20 was added to assist in disrupting the hydrophobic barrier between *Aspergillus* spores and H_2O and to make the solutions more uniform in terms of spore suspension. Tween 20 was added to all treatments to ensure consistency between treatment effects. The fungal inoculum was screened through a polypropylene mesh cone to remove agar and mycelial debris and shaken to disperse spores thoroughly.

Spore concentrations were determined by hemacytometer counts. Natural spore count trials used non-dilute concentrations of spores from culture plates of both test fungi. Initially, *Alternaria* counts averaged 0.606×10^6 spores/mL in 30 mL H_2O while *Aspergillus* spore counts were much higher at 22.8×10^6 spores/mL. A dilute spore solution (1.00×10^6 spores/ mL in H_2O) of both fungi was used for inoculation in the case of precise tests. Scarified lots of seed were flood inoculated in cone sieves by recycling spore solutions three times. Use of flood inoculation ensured complete exposure of seeds to the fungal inoculum by momentary suspension of seeds in solution. The thin films of water surrounding the seeds after flood inoculation were critical for spore retention. A variation on the direct inoculation tests was performed in which unscarified lots of seed were inoculated in the natural spore count solution to determine whether or not scarification was crucial to the fungal interactions.

Laboratory *in vitro* germination studies were replicated three times for the natural and four times for the precise spore inoculations. Each replication consisted of a randomized complete block design, with two treatment groups (*Alternaria* and *Aspergillus*) and one control group (H_2O) within each of four blocks. Groups of twenty

seeds were randomly selected from treated lots and assigned to each block consisting of a stack of randomized Petri dishes, one for each treatment. Seed was placed on 8.30 cm (diameter) round steel blue seed germination blotters (Anchor Paper Company) wetted with 7.00 ml reverse osmosis H_2O to insure maintenance of the humid conditions necessary for seed germination and fungal development. Petri dishes were sealed with Parafilm® to help maintain moisture. Blocks were lined up and kept in a dark growth chamber at 15.0 ± 2 °C. Daily cumulative germination counts were recorded through day 8 or 11 depending on the inoculum pressure (natural vs. precise) with germination defined as visible extension of the radical outside of the seed coat.

Growth chamber *in vivo e*mergence studies, replicated 3 times, were setup utlizing a randomized complete block design with the treatments (*Alternaria* and *Apsergillus*) and control (H_2O) contained within each of 4 or 5 blocks. Each block consisted of 1 tray of 66 elongated root training cells sterilized with sodium hypochlorite and filled with prepackaged soil. Groups of 18 seeds were selected from each treatment and assigned at random to groups of 18 cells (block) with one seed per cell. Three treatment groups (blocks) were randomly assigned to sections of every tray with a spacing row of unplanted cells between each treatment to help prevent cross contamination. Trials were conducted with assigned planting depths of 0.065 to 0.65 cm.

In vivo trials were conducted in conditions of increasing environmental complexity. The two environmental growth chamber trials involved planting in four blocks enclosed in a humid chamber, kept at 90 to 100% relative humidity, for 1 to 2 weeks at 21°C in a 12 hr light/dark cycle with bottom watering. After 1 to 2 weeks the chamber was removed from surrounding the blocks and the seedlings exposed to the

normal environmental growth chamber conditions of 21°C in a 12 hr light/dark cycle with bottom watering. Cumulative germination counts were recorded daily through 30 days.

Greenhouse trials, conducted in five blocks were kept under a mister without bottom heat for 7 days to assist with the maintenance of soil moisture after which they were moved to a dry bench. Blocks were lined up north to south to minimize variability in temperature and exposure to light among blocks over each 24 hr period. Plants were watered every 3 to 4 days or as needed with no supplemental nutrients added. Daily germination counts were recorded through day 9 and cumulative germination was recorded at approximately one month. Seedling death was counted as a measure of survival throughout the continuation of the tests.

Field trials included preliminary trials planted in the Fall of 2005 and the Spring of 2006 at two and three sites, respectively. A combination of 6 treatments was used including: *Aspergillus* (5 hr and 12 hr), *Alternaria* (5 hr and 12 hr) and control (scarified and unscarified) lots. Seed treated during preliminary trials was inoculated and dried for 5 and 12 hours before refrigeration. Control seed was surface planted with a specialty seeder while treated seed was hand planted to prevent cross contamination. Emergence was counted after 6 months for the fall plantings and 2 months for the spring plantings. All field tests were a randomized complete block design consisting of 3.0 meter rows, with the number of rows per block depending on the treatment combinations, and 150 seeds per treatment in 5 blocks.

Trials conducted in the fall of 2006 and Spring of 2007 were surface planted at 3 sites. Treated seed was dried for 12 to 24 hours to encourage fungal attachment after

which it was refrigerated for 3 weeks prior to planting. A tractor mounted specialty seeder was used for planting all control and treated seed. The implement was sterilized with 98.0 % ethanol between treatments to eliminate cross-contamination. Emergence counts were taken at 6 months for fall plantings and 3 months for spring plantings.

Though field sites were selected according to availability they encompassed a wide range of conditions representing different levels of complexity in elevation, moisture and temperature throughout north-central Utah. Three Utah sites selected for field trials were: Fountain Green, Nephi, and the Brigham Young University Agriculture Research Farm near Spanish Fork.

The site near Fountain Green is nestled in the mountain valleys leeward of the first group of mountains in the Wasatch range at an elevation of approximately 1800 meters on cultivated ground. This site receives moisture in the form of snow and rain with approximately 2.50 cm of precipitation per month during the spring season. Temperatures range from -1.0 to 10.0 °C during initial spring months (March and April) when *A. utahensis* seeds begin to germinate. The Nephi site is located in an open valley floor at a lower elevation of approximately 1500 meters on cultivated ground. This site receives moisture in the form of snow and rain with approximately 3.80 cm of precipitation per month during the spring season. Temperatures range from 1.6 to 10.0 °C during initial spring months noted above. The BYU research farm is on the windward side of the Wasatch Range at a lower elevation of approximately 1300 meters on cultivated ground. This site receives moisture in the form of snow and rain with approximately 5.1 cm of precipitation per month during the spring season. Temperatures range from 3.3 to 12.8 °C during initial spring months noted above.

Seedling mortality was useful in determining detrimental effects of fungi on germinating seed. Select seedlings showing a variety of symptoms ranging from damping off to blackened stalks were removed from greenhouse tests. Plant material was pressure rinsed in reverse osmosis H_2O to remove organic matter and soil, sterilized in 10.0 % sodium hypochlorite for 30 seconds, and rinsed in reverse osmosis H_2O for 30 seconds. Seedlings were cut into sections and plated on 1/3 strength potato dextrose agar (1/3 PDA) and cornmeal agar (CMA) culture media in an attempt to isolate fungi. Cuts were made peripheral to symptomatic areas of infection ensuring isolation of parasitic microbes. Execution of aseptic technique in a laminar flow hood decreased contamination during plating. Mortality was monitored and recorded in greenhouse and growth chamber trials to provide a statistical measure of seedling survival.

All data involving germination over time were analyzed using Poisson regression. Significance of the terms is determined by the probability that the difference between mean counts of each coefficient compared to the intercept is zero (null hypothesis). The Chi square values produced by the model are a measure of how close the means come to proving the null hypothesis. A large Chi square value indicates greater probability that the mean of the control (intercept) is different from the treatments (coefficients). A probability value (p value) is calculated from the relationship between control and treatment values projecting the degree of difference between the means. An alpha level of 0.05 is used to determine statistical significance with a p value of < 0.05 labeled significant.

The experimental design, a randomized complete block design, allowed statistical capture of the variation that existed as a result of subtle differences in temperature,

location and other variable within a test location, environment or site. Analyses showed significance in most independent variables (i.e. block, test, site, year, etc.) and the interactions between these variables indicating their usefulness and necessity in describing the variability that existed among treatments. In the case that statistical differences ($p < 0.05$) existed between treatments it is essential to express those differences as a function of the significant independent variables like block, test, site and year, keeping them in the model. In the case of the data described in this paper the intercept (scarified control) is the base value to which all other treatments are compared.

Random assignment of seed to their prospective treatments allows causal inferences to be made. Responses identified as statistically significant are implicit in the hypothesis that fungal treatment has an accelerating effect on the germination of this hard-seeded species. Although inferences to the general population of *A. utahensis* plants throughout their native range cannot be made, there is a definite confirmed response for the seed lot used in this study gathered from the Spanish Fork, Utah site.

Results

The natural and precise spore count germination tests indicate a significant accelerated germ response when seed was treated with *Alternaria* and *Aspergillus* fungi (Figure 1). Natural spore count trials exhibited a highly significant difference between control (5.0 %) and treated seed, with *Alternaria* seed germinating at 95.0 % ($P < 0.0001$) and *Aspergillus* germinating at 55.0 % ($P < 0.0001$). Comparison of the treatments yielded significant differences ($P < 0.0001$) between fungal treatments with *Alternaria* exhibiting 40.0 % more germination than *Aspergillus. The* precise inoculation yielded less pronounced effects with control seed exhibiting minor germination (10.0 %),

Alternaria germinating at 75.0 % and *Aspergillus* yielding 45.0 % germination. Both treatments were highly significant with p values of ($P < 0.0001$) (Figure 1). Analysis of differences between treatments in the precise inoculation showed highly significant differences ($P < 0.0001$) with 30.0 % greater germination in *Alternaria* than *Aspergillus.*

The variation on direct inoculation in which the natural spore count solution was used, without replication, to test the response of unscarified seed showed that control seed was significantly different ($P < 0.0001$) with no germination (0.0 %) from *Aspergillus* treated seed with 35.0% germination. *Alternaria* treated seed also showed similar significant differences when compared with the control yielding 25.0% germination (Figure 1).

Growth chamber trials indicate significant differences among the treated and control seed (Figure 2). Combined analysis of the trials yielded significance differences ($P < 0.0001$) between the control treatment (44.0 %) and the *Aspergillus* treatments (11.0 %) (Figure 2). Further analysis shows insignificant differences ($P = 0.5268$) in the relationship between control and *Alternaria.* Significant differences exist between the two fungal treatments ($P < 0.0001$) with *Alternaria* germinating 33.0 % higher than *Aspergillus*.

Greenhouse trials supported *in vitro* trials in that the control seed germination was significantly lower than the treated seeds. *Alternaria* and *Aspergillus* treated seed had significantly different ($P=0.0106$) germination responses with *Alternaria* seed germinating at 55.0 % and *Aspergillus* at 50.0 %. The treatment differences resulting from the fungi were highly significant when compared with the control ($P < 0.0001$) (Figure 2).

Field trials planted in the fall of 2005 resulted in significantly ($P < 0.0001$) lower germination for the treated seed than control seed (Figure 3). Treated seed had less than 6.0 % of seeds germinate for both the *Alternaria* and *Aspergillus* in contrast to 36.0 % germination by the scarified control seed ($P < 0.0001$) (Figure 3). Spring 2006 trials had 0.0% germination for all seed treatments including the scarified control seed (Data not shown).

Fall 2006 field trials were different from 2005 because seeds treated with *Alternaria* and *Aspergillus* had significantly higher ($P < 0.0001$) germination, at least 3.0 % and 7.0 % higher, respectively, over the scarified control seed which germinated between 4.0 % and 39.0 % depending on location (Figure 4). The Nephi site yielded significant differences in germination when treated seeds were compared to control seed (17.3 %) with a 12.0 % increase for the *Aspergillus* treatment (29.3 % germination) and a 3.3 % increase for *Alternaria* (20.7 % germination) (Figure 4). *Aspergillus* also showed significant differences ($P < 0.001$) when compared with *Alternaria,* germinating 8.6 % over the *Alternaria* treatment. Fountain Green showed similar increases ($P < 0.0001$) over the control (4.0 % germination) with a 7.3 % increase for *Aspergillus* treated seed and a 4.0 % increase for *Alternaria* (Figure 4). Data for this site shows little practical difference (2.0 %) between *Alternaria* and *Aspergillus* treatments ($P < 0.0001$). Data from the BYU research farm showed the *Alternaria* treatment having a highly significant negative correlations ($P < 0.0001$) with the scarified control seed while *Aspergillus* treated seed showed insignificant differences ($P = 0.2616$) when compared with the control seed. *Aspergillus* showed a 12.0 % increase over the *Alternaria* treatment (Figure 4). Unscarified control seed did not germinate at a significantly

greater rate than the scarified control seed at any of the field locations (Figures 3 and 4). The highest germination of unscarified seed was 5.3 % in the fall of 2005 as seen in combined Fountain Green and Nephi data and compared with none in fall of 2006 data (Figures 3 and 4). The soaked control, a treatment assuming the presence of leachable inhibitory substances, did not have increased germination over the scarified control; the highest germination rate was 23.3 % still 16.0 % below the control (Figure 4).

Mortality monitoring for seedling death in the presence of the test fungi showed that the values in soil under nearly optimal conditions experienced in the greenhouse yielded 100 % survival for all treatments. The growth chamber trials showed significant loss of seedlings with 50.0 % survival for control seed, 20.0 % survival for *Alternaria* seed and 0.0 % survival rate for *Aspergillus* seed.

Discussion

Barneby (1964) describes *Astragalus utahensis*, common name Utah milkvetch, as a perennial xerophyte distributed throughout the western United States, Northern Mexico and Southwestern Canada with an effective elevation ranging from 1250m to 2130m. It is noted (Barneby, 1964) that this tap-rooted, herbaceous plant is found flourishing on calcareous soils located on dry stony hillsides, open gravelly banks and river terraces and is usually associated with sage, oak brush and juniper.

Under these arid conditions, plants such as *Astragalus utahensis* have evolved mechanisms to control germination that can be considered exogenous, described by Nikolaeva (1977) as mechanical and chemical dormancy. These include the presence of chemical inhibitors (Baskin and Quarterman, 1969; Evenari, 1949; Green, 1973; Ziemkiewicz and Cronin, 1981), seed coat impermeability (Caudle, 1968; Evenari, 1962;

Miklas et al., 1987; Rolston, 1978; Ziemkiewicz and Cronin, 1981) and, more *Astragalus* specific, the possible presence of a thin, but strong second seed coat (Baskin and Quarterman, 1969). Miklas et al. (1987) also acknowledged the elements of this dormancy, by describing the hard-seeded nature of *Astragalus cicer* and its effect on imbibition and eventual germination. However, *Astragalus utahensis* holds potential for use in restoration because of xeric characteristics, proteinaceous nutrition, and non-toxic chemistry if germination barriers can be overcome.

The influence of microorganisms on the breakdown of seed dormancy is not a completely unstudied subject. Studies dealing with this phenomenon reach as far back as 1934 when Pfeiffer (Pfeiffer, 1934) suggests that fungi are essential for the germination of *Symphoricarpos racemosus* seeds by means of compromising seed coat integrity.

In vitro trials in this study relate treatment and inoculum pressure to germination (natural and precise germination tests). Results of the in-vitro germination tests show that the differences between treated and non-treated seed are substantial with *Aspergillus* and *Alternaria* treated seed accelerated at least 35.0 % and 90.0 %, over the control seed.

Other researchers have reported instances of accelerated germination including Koaze (1957) in which a culture filtrate of *Streptomyces* sp. S-580 was reported to increase germination in rice seeds, Niemi and Haggman (2002) who showed promotion of germination in somatic embryos of Scots pine, *Pinus sylvestris*, when placed (contact free) in the same petri dish with *Pisolithus tinctorius*, a fungus, and Rheeder et al. (1990) who report a positive correlation between *Fusarium subglutinans*, as well as undefined other fungi, and corn germination. Still other studies report interactions that accelerate seed germination Guttridge et al. (1984) who report significant germination increases of

seeds inoculated with *Ulocladium charatarum* fungus (74%, 7%) over the control (38%, 2%) for two varieties of strawberry. Morpeth and Hall (2000) also report significant increases in germination of *Rosa corymbifera* 'Laxa' seed after being enhanced with a commercial compost activator. Researchers in this case concluded that natural microbial loading was necessary for germination. Finally, Schafer and Kotanen (2004) report enhanced germination of *Solidago nemoralis* and *Verbascum thapsus* in the presence of fungi including *Alternaria*.

Growth chamber trials resulted in similar germination patterns among the treatments. Although the growth chamber was the ideal place to test this response, there was a confounding effect inherent to the initial germination period where an enclosed humid chamber was used to promote germination. The plastic enclosure allowed soil moisture content to remain elevated enough to facilitate germination, but these conditions also increased disease in the seedlings by the test fungi as well as other microorganisms present in the soil surrounding environments. Inconsistent exposure of seeds to these conditions ranging from 1 to 2 weeks added a degree of variability to the confounding effects as related to the disease factor. The control and *Alternaria* seed were not significantly ($P = 0.5268$) different from each other with a germination rate of 44 % while the *Aspergillus* seed exhibited significantly ($P < 0.0001$) lower germination at 11.0 %.

Greenhouse trials were conducted during all seasons of the year, thus experiencing relatively mild conditions regardless of the season. Data from the final greenhouse trial showed a common response between treatments in relation to the control. *Aspergillus* and *Alternaria* treatments performed well germinating at least 30.0 % over the control. The results of this test are interesting and show an evolution of

the germ response as it is exposed to environments involving increased variability.

Field trials were performed because it was necessary to test the usefulness of the fungi in an applied system. Trial results from the fall of 2005 showed erratic and indeterminate germination patterns between seed treatments with control seed (36.0 %) outperforming treated seed (0.5 to 6.0%); spring of 2006 trials germinated at 0.0 %. Much of this inconsistency was likely due to the difference in planting methods because the controls were planted by mechanical means and the treated seed was planted by hand to avoid cross contamination of the planting implement. Even though great efforts were made to mimic the depth and compaction from the planter, differences in seed placement and soil contact appeared to have a considerable influence on preliminary germination trial regardless of treatments. It was decided that future field tests would use the specialty seeder for all plantings to minimize variability. Adjustment of the planting regime during fall 2006 and spring 2007 to include use of the planting implement for all treatments yielded distinguishable differences between control and treated seed. One interesting difference was that *Aspergillus* significantly ($P < 0.0001$) outperformed *Alternaria* by at least 3.0 % and outperformed the scarified control seed by 15.0 %.

Planting sites were compared individually across years, fall 2006 and spring 2007, in order to detect effects of planting time on performance. Comparisons yielded insignificant practical responses for fall 2006 and spring 2007 combined statistics on each site (0.0 % germination for all treatments). This comparison of individual sites across years was influenced heavily by the spring 2007 trials. The influence was due in part to shorter germination time (2 months) and the limited exposure to moisture. Fall plantings

allowed for a longer germination window (6 months) and exposure to more moisture during early spring months.

The analytical approaches inherent to the randomized complete block design and analysis using Poisson regression enabled capture of the most productive planting times, indicating the fall plantings for the Fountain Green and Nephi sites to be highly significant when treatment values were compared with the controls. Statistical analysis of these tests combined showed a highly significant response ($P < .0001$). The scarified control seed germinated at 14.7 % with the respective treatments, *Aspergillus* and *Alternaria* germinating at 29.3 and 19.3 %.

Differences among the field trials sites appeared to influence the germination of *A. utahensis*. The elevation and location of the Fountain Green site served to provide the plantings with a more gradual supply of moisture over the summer months. This location and associated attributes would allow for greater infiltration of soil moisture and in this case possibly greater leaching of the seed. Despite these benefits however these conditions would also keep temperatures below those necessary for metabolic activity longer than seedlings might experience in lower valley locations found at Nephi and the BYU research farm.

The Nephi site, though ideal in terms of temperature, was lacking in the moisture retention necessary to allow seed germination. This sight looses snow cover early due to elevation and location in the main valley resulting in limited soil moisture when temperatures are conducive to germination. It is likely that the moisture received by this location during the early spring is heavy and rare because of its isolation in the valley. This would encourage inefficient use of the early spring moisture by the seeds

discouraging germination.

The BYU farm site could be considered the most temperate site exhibiting conditions conducive to germination. With snow and rain through the fall and winter and into spring this soil would be rich in moisture and spring temperatures conducive to germination lengthening the germination window. However, warm temperatures and fall moisture might result in premature establishment and priming of the seed causing it to freeze during the harsh winter months. Results of the field trials show that *A. utahensis* had significantly better ($P < 0.0001$) germination when fall planted on Nephi and Fountain Green sites. Such planting yielded a combined germination rate of 5.0% and 15.0% germination over the control for *Alternaria* and *Aspergillus*, respectively.

Attempts to grow this arid adapted tap-rooted species on cultivated ground (i.e. BYU farm) might set the seedlings at a disadvantage. When put in terms of the dry, well drained, shallow soils where this Utah native flourishes, the deep, heavy cultivated soil might not be ideal conditions. As evidenced from observations made in preliminary Greenhouse trials, excessive moisture, even in the form of daily watering caused stunted plant growth.

Given the potential parasitic nature of these fungi involved in the testing it is important to note the extent to which these characteristics were manifest in the course of the experiments. Mortality monitoring showed a relatively consistent response for control seed yielding none of the test fungi in culture. Other microbes were detected, none of which were identified. *Aspergillus* treated seed showed the presence of the test fungus in 3 of the 5 seedlings collected with symptoms including damping-off and water soaking. *Alternaria* treated seed showed the presence of the test fungus in 5 of the 6

infected seedlings. Symptoms of the seedlings selected for culturing included blackening of the radical and cotyledons, damping-off as well as wilting. The survival data for the greenhouse trials shows that given the right conditions the seedlings are able to survive suffering low mortality rates of, 0.0 % and 20.0 % for *Aspergillus* and *Alternaria*, respectively.

The accelerated germ responses exhibited in these tests could well be a function of the environment in which the trials were conducted. As previously discussed *Alternaria* proliferates at mild temperatures and high moisture content, conditions common to the design of the *in vitro* experiments. *Aspergillus* may have performed poorly in this environment because although able to adapt to a wide range of temperatures it is described as performing best in dry, droughty conditions (Cotty et al., 1994). An evolution of responses exhibited by both treatments is supported by the data gathered from all phases of testing (Figure 5). *In vitro* platings were dominated by *Alternaria* with at least 65.0 % germination over the control. Greenhouse germ responses showed consistently similar responses by *Alternaria* and *Aspergillus* with 33.0 % to 38.0 % germination over the control. The field trials however show that *Aspergillus* treated seed responses dominated the field trials ranging from 7.0 % to 12.0 % germination over the control. While reversal of performance by *Alternaria* may correlate to sub-optimal field temperatures during the spring in contrast such conditions were probably more suited to growth and development of *Aspergillus*.

If seeds harness the physical and chemical mechanisms of fungi to promote breakdown of the seed coat, or germination, and then establish and survive as seedlings, the hard-seeded nature of such species could be circumvented temporarily allowing the

accelerated and uniform germination necessary in restoring native plants to a post-disturbance ecosystem fraught with competition from invasive species. The results of the experiments described in this paper, both *in vitro* and *in vivo* trials, indicate that these processes significantly enhance the germination of seeds treated with fungi.

Conclusion

Unless dormancy is altered genetically or by some other means, arid plants exhibiting hard-seeded characteristics are at a disadvantage in terms of establishment and survival in an environment as raw and open as the post disturbance ecosystem. Genetic manipulation through plant improvement or removing dormancy characteristics by breeding them out of the species, though effective, proves to be a long and arduous process. Not only does such work require years of testing and certification but attention must be given to preserving life history rudiments engendering its competitive fitness in a post-disturbance establishment regime that are critical to its long-term survival. Use of an impermanent treatment aiding in a temporary morphological or physiological disruption of exogenous dormancy would necessarily be instrumental in furnishing these species with a competitive edge over other invasive plants establishing in the same conditions.

The data gathered during the course of this study shows a definite accelerated response of *A. utahensis* seed to fungal treatments *in vitro*. The response evolves as it is tested in environments that exhibit a continuum of greater variability. While *in vitro* tests favor *Alternaria*'s response, the greenhouse tests show a uniform performance in both treatments. With the increased variability present in the field tests *Aspergillus* dominates the response. Not only do these data confirm an accelerated response but the viability of

the response is proven in the field. It is important to note that the evolution of this response correlated directly with the ecology and life history of the fungi involved. In an environment where moisture and temperature were controlled at optimal levels *Alternaria* thrived. True to the robust nature of *Aspergillus*, seeds treated with this fungus were at a competitive advantage, when compared with the other treatments, in the more extreme conditions of a field environment.

The beneficial fungal-seed interactions presented manifest the efficacy of developing these associations as significant dormancy contravention treatments in hard-seeded species. Although the methods and results described do not surrogate molecular studies focused on discovery and breeding with respect to dormancy, beneficial interactions might provide managers with an effective way of rapidly and efficiently restoring hard-seeded native species to disturbed lands. Though the ultimate goal should be defining these factors, so as to gain a complete understanding of the mechanisms involved, the responses established support this objective by providing a statistical foundation from which the mechanistic responses can be derived through further experimentation. The real-world response established here not only provides the basis of consistency from which the mechanisms involved can be derived but it also provides practical application to this phenomenon with regards to parties involved in the restoration of native species on disturbed lands.

Literature Cited

Agarwal, V.K. and J.B. Sinclair. 1997. Principles of seed pathology. 2nd eds. CRC Press. Boca Raton, Florida.

Agrios, G.N. 2005. Parasitism and disease development. In: Plant Pathology. 5th edition. Elsevier Academic Press, New York, pp 77-104.

Barneby, R.C. 1964. Atlas of North American *Astragalus*. Memoirs of the N.Y. Botanical Garden vol. 13 1188 pp.

Baskin, C.C. and J.M. Baskin. 1998. Seeds: ecology, biogeography, and evolution of dormancy and germination. Academic Press. San Diego, CA USA.

Baskin, C.C. and E. Quarterman. 1969. Germination requirements of seeds of *Astragalus tenneseensis*. Bull. Tor. Bot. Club 96: 315-321.

Bibbey, R.O. 1948. Physiological studies of weed seed germination. Plant Physiology 23: 467-484.

Caudle, C. 1968. Studies on the life history and hydro-economy of Astragalus tenneseensis (Leguminosae). Ph.D. dissertation, Vanderbilt University, Nashville, TN.

Cotty, P.J., Bayman, P., Egel, D.S. and K.S. Elias. 1994. Agriculture, aflatoxins, and *Aspergillus*. In: The genus Aspergillus from taxonomy and genetics to industrial application. Powell, K.A., Renwick, A. and J.F. Peberdy eds. Plenum Press, NewYork p 1-27.

Donald, C.M. 1963. Competition among crop and pasture plants. Advances in Ag. 15: 1-118.

Evenari, M. 1949. Germination inhibitors. Bot. Rev. 15: 153-194.

Evenari, M. 1962. Plant physiology and arid zone research. Arid Zone Res. 18:175-195.

Green, T.W. 1973. Factors affecting the ecology of *Astragalus libarius* and *Astragalus utahensis* with emphasis on role of insects. Ph.D. dissertation Utah State University. 143 p.

Guttridge, C.G., Woodley, S.E., and T. Hunter. 1984. Accelerating strawberry seed germination by fungal infection. Annals of Botany 54(2): 223-230.

Koaze, Y. 1957. Germination promotants for plant seed, produced by microorganisms. Bull. of the Ag. Chem. Soc., Japan 22: 91-97.

Miklas, P.N., Townsend, L.E., and S.L. Ladd. 1987. Seed coat anatomy and the scarification of Cicer Milkvetch seed. Crop Sci. 27: 766-772.

Morpeth, D.R. and A.M. Hall. 2000. Microbial enhancement of seed germination in *Rosa corymbifera* 'Laxa'. Seed Sci. Res. 10: 489-494.

Niemi, K. and H. Haggman. 2002. *Pisolithus tinctorius* promotes germination and forms mycorrhizal structures in Scot's ine somatic embryos in-vitro. Mycorrhiza 12: 263-267.

Nikolaeva, M.G. 1977. Factors controlling the seed dormancy pattern. In: The physiology and biochemistry of deep seed dormancy. A.A. Khan eds. North Holland Publishing Co., New York pp 51-74.

Pfeiffer, N.E. 1934. Morphology of the seed of *Symphoricarpos racemosus* and the relation of fungal invasion of the coat to germinative capacity. Cont. Boyce Thompson Institute 6: 103-122.

Rheeder, J.P., Marasas, W.F.O. and P.S. Vanwyk. 1990. Fungal associations in corn kernels and effect on germination. Phytopathology 80: 131.

Rolston, M.P. 1978. Water impermeable seed dormancy. Bot. Rev. 44: 365-396.

Rotem, J. 1994. The genus *Alternaria*. The American Phytopathological Society, St. Paul Minnesota USA.

Schafer, M. and P.M. Kotanen. 2004. Impacts of naturally occurring soil fungi on seeds of meadow plants. 2004. Plant Ecol. 175: 19-35.

Spooner, B. and P. Roberts. 2005. Fungi. Harper Collins Publishers, London.

Stiles, E.I. 1948. Relation of water to the germination of corn and cotton seeds. Plant Physiology 23: 201-222.

Vasquez-Ramos, J.M. 2003. The cell cycle and seed germination. Seed Sci. Res. 13(2): 113-130.

Ziemkiewicz, P.F. and E.H. Cronin. 1981. Germination of seed of three varieties of Spotted Locoweed. J. Range Man. 34: 94-97.

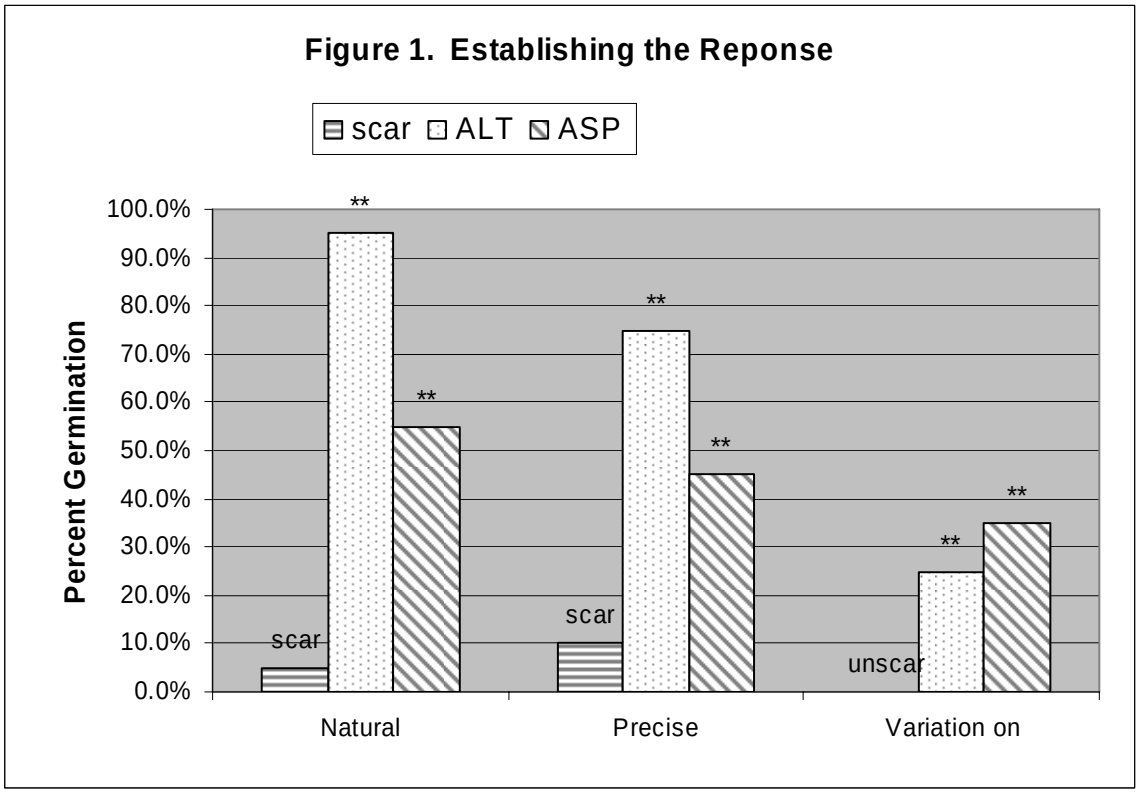

Figure 1.1. Natural and precise spore count germination tests with seed treated by *Alternaria* and *Aspergillus* inoculum.

** P value < 0.0001

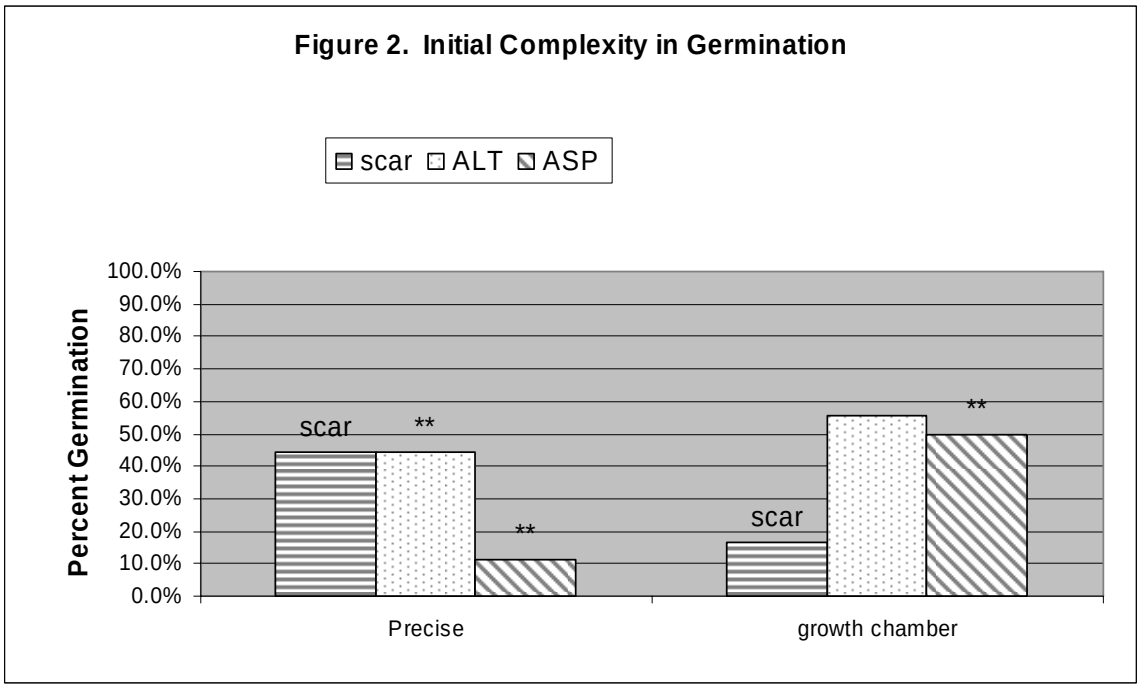

Figure 1.2. Growth chamber and greenhouse trials showing treated and control seed.

** $P < 0.0001$

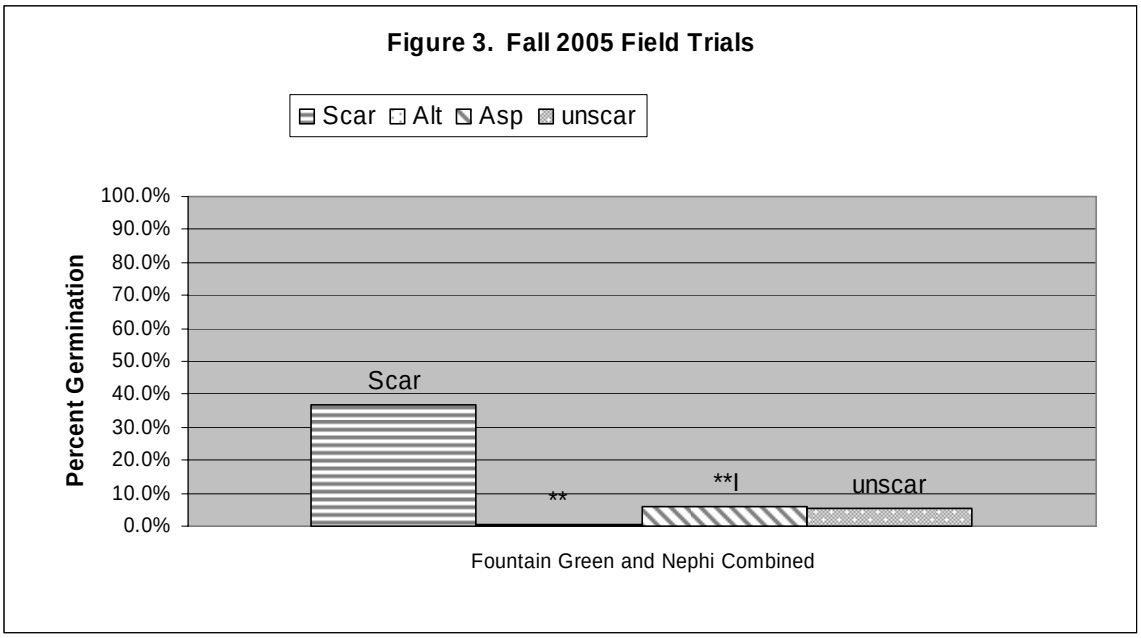

Figure 1.3. Field trials planted in the fall of 2005 at Fountain Green and Nephi.

** $P < 0.0001$

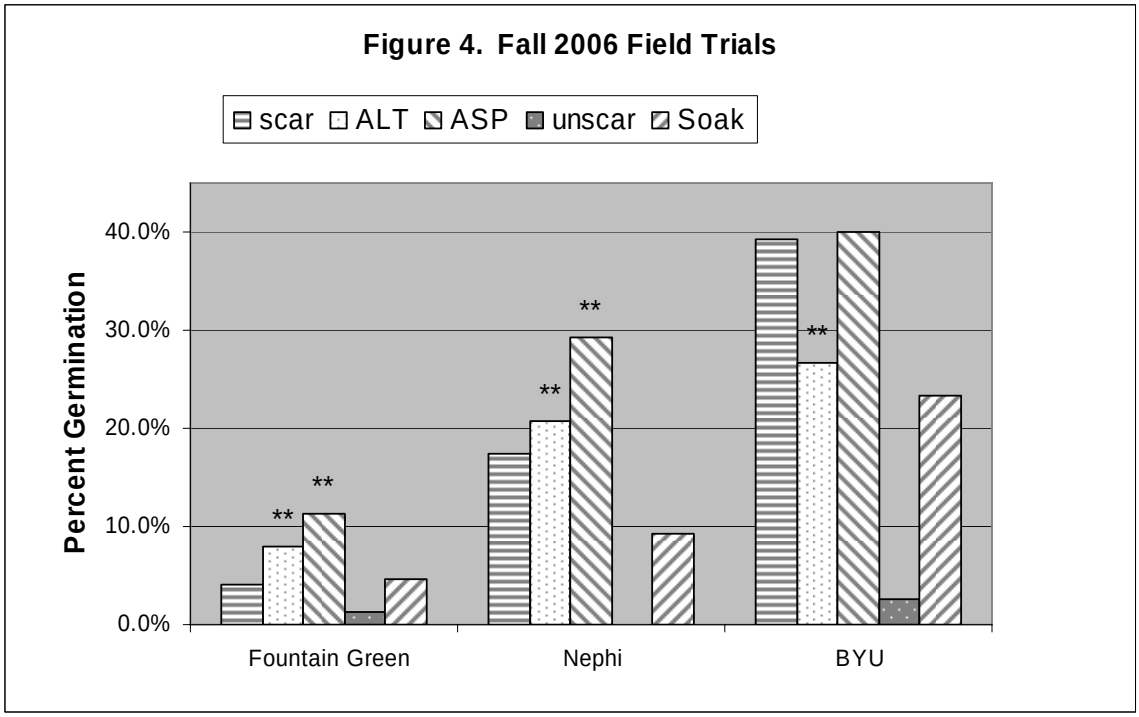

Figure 1.4. Fall 2006 field trials at Fountain Green, Nephi and the BYU research farm.

** $P < 0.0001$

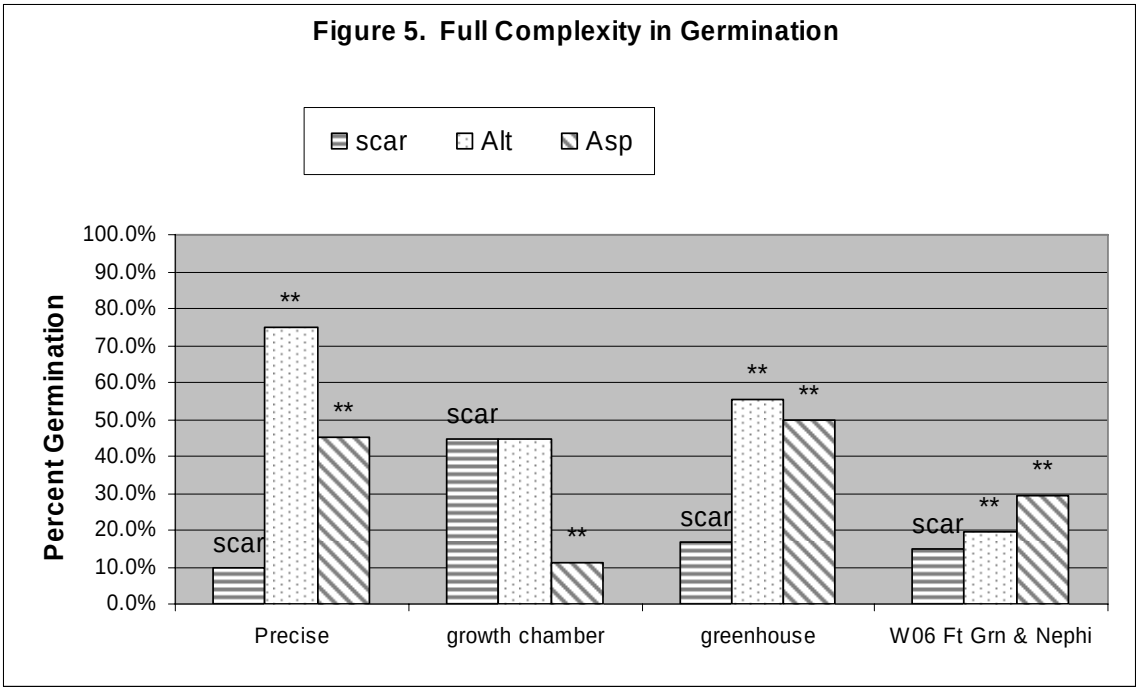

Figure 1.5. Evolution of responses exhibited by Alternaria and Aspergillus treatments as variability in complexity ranges through all locations in this study.

** $P < 0.0001$

Chapter 2

Seed Germination as Influenced by Microorganisms and the Associated Natural Resource Management Implications

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Abstract

Rapid seed germination is essential to the establishment and survival of many native species. Dormancy, a major component governing germination patterns, is critical to the survival of species under certain undesirable environmental conditions. Dormancy characteristics governing seed germ responses can, however, inhibit germination even under ideal conditions. Microbial activity in relation to seed germination involve the break down of exogenous dormancy barriers in turn allowing the seed to germinate. Increased microbial activity resulting from the exogenous relationship between microorganisms and seeds in the spermosphere during germination may result in beneficial, harmful or non-productive interactions. A proposed paradigm for studying these interrelationships characterizes these interactions as being *directly* or *indirectly* correlated with the microbial mechanisms affecting seed germination. *Direct* correlations involve close associations between microbes, their mechanisms of action and seed germination. *Indirect* correlations involve stepwise, cause and effect responses in this association between seed and microbe. Beneficial effects of microbes, those which accelerate normal germination patterns of seeds, can be defined as being *directly* correlated with seed germination. Harmful effects, those which suppress or alter normal seed germination characteristics, are defined as having *direct* negative correlations to seed germination. Nonproductive effects of microbes on seeds may ultimately result in beneficial, harmful, or neutral influences on germination, but they are defined as being indirectly correlated with seed germination because they do not influence it directly but rather alter the environment in the spermosphere. Analysis of 35 references assembled from a total of 25 studies, describe interactions between seed and microbe as they are

related to germination. The abundance of data supporting harmful and nonproductive interactions shows a negative trend in the ability of seed and microbe to optimally coexist in the spermosphere. However, established seed-microbe interactions prompting quicker germination offer a firm basis for analyzing the potential benefit and efficacy of developing these associations as significant dormancy contravention methods.

Introduction

Seed germination (Vasquez-Ramos et al., 2003) is the initiation of processes involved in metabolism and development in the dormant seed. Germination is pivotal in the life cycle of a plant, without it mechanisms of life would not proceed (Stiles, 1948). The quicker germination occurs the speedier the establishment characteristics of the plant can take over and work to ensure survivability. Bibbey (1948) points to the quick germination response of weed seeds as the characteristic, independent of the species competitive abilities and seed production, which allows them to maintain viability in the face of disturbances. This faculty of invasive species frequently hampers the revegetation of disturbed lands with native species in post-disturbance ecosystems and thus constitutes a formidable barrier to restoration.

In an undisturbed environment, seed dormancy is ecologically significant because it delays germination until conditions are right for the establishment of seedlings (Green, 1973). This argument is strengthened when Green (1973) suggests that dormancy is critical to the lifecycle of plants living in environmentally unstable conditions because it allows for the maintenance of an annual seed bank. This germ bank allows for relatively keen flexibility in terms of rapid response in ideal conditions and continued viability during perilous seasons. In short, dormancy or control of seed germination is critical to

the survival of species under certain undesirable environmental conditions.

Dormancy is a complex interaction between external environmental and inherent internal germination barriers that must be breached if germination is to occur (Baskin and Baskin, 1998). This inability of a seed to resume metabolic processes despite the source of inhibition is directly linked to reactions taking place at a cellular level. Growth of a seedling made possible by completion of the four phases of the cell cycle (G1, S, G2 and mitosis) relies on a complex series of responses including DNA replication, protein synthesis, and positive or negative feedback inhibitions (Vasquez-Ramos, 2003). Interruption or supplementation of these processes by external or internal impetus, whether chemical or environmental (Matsushime et al., 1991), at the gate of the cycle (Xiong et al., 1992), decides whether inhibition or acceleration of the ge rmination response occurs. Managing the cell cycle at its gate by considering the presence or absence of these stimuli is the key to understanding seed dormancy and germination.

Nikolaeva (1977) made two solid distinctions within the context of organic dormancy, or inherent internal seed germination barriers, grouping germination according to the barrier mechanisms involved, endogenous or exogenous dormancy. Endogenous dormancy is related to the physiology of the seed and its developmental characteristics, while, exogenous dormancy is related to the presence of chemical inhibitors, impermeable seed coats and growth restricting structures; the latter is one of the instrumental factors in seed-microbe interactions. Complimentary studies by Green (1973) and Caudle and Quarterman (1968) acknowledged the elements of exogenous dormancy when they independently reported substantially low germination rates, over a 9-month time course study in *Astragalus utahensis* and *A. tennesseensis* seeds.

Miklas et al. (1987) also acknowledged the elements of this dormancy, by describing the hardseeded nature of *A. cicer* and its effect on imbibition and eventual germination. Williams and Elliot (1960) provide additional support for the exogenous concepts discussed when they insisted that survivability, and stand establishment of legumes is tied closely with seed coat impermeability.

Microorganisms and their Influence on Seed Germination

Microbial interactions with plant tissue as described by Agrios (2005) usually begin with production of inoculum, typically spores, through sexual or asexual means. Dissemination occurs and tissue is inoculated by cells or spores through means of direct contact; inoculation is followed by penetration, infection and colonization. The processes of penetration and infection are accomplished by a combination of two mechanisms: physical penetration and chemical breakdown of host tissue. Physical infections begin with growth of the associated cells or spore(s) and continue with incursion into wounds, pores or other natural openings in the tissue. Chemical infections begin with growth of the cell or spore and release of enzymes that break down the host tissue, allowing the microorganism to grow into the host and absorb nutrients. Regardless of physical or chemical mechanisms, infection and colonization continue, releasing enzymes that facilitate break down of host tissue, allowing further absorption of carbon and other nutrients from the surrounding material as well as providing further access into the host .

The influence of microorganisms on germinating seeds has been studied on a limited basis. One of the earliest studies dealing with this phenomenon published by Pfeiffer (1934) suggested that fungi are essential for the germination of *Symphoricarpos racemosus* seeds because the microorganism compromised seed coat allowing emergence

of the radical. In a more recent study Nelson (2004) suggests that seeds have evolved in close association with a very diverse population of microbes in the environment surrounding the seed, also known as the spermosphere. Nelson (2004) attributes the proposed relationship, in part, to the conditions perpetuated as a seed begins its metabolic processes. One major component of this dynamic interaction of microbes in the spermosphere is the presence of seed exudates (Nelson, 2004). These soluble carbon sources (Lynch, 1978) are composed of natural cell components including sugars, amino acids, and proteins, which serve as stimulants for microbial growth (Nelson, 1990). They encourage the microbes to arrest their natural state of dormancy, caused by low nutrient availability, and begin growth and activity (Lynch, 1978; Nelson, 1990; Nelson, 2004). Microbial activity includes the break down of exogenous seed dormancy barriers in turn allowing the seed to germinate.

Increased microbial activity resulting from the exogenous relationship between microorganisms and seeds in the spermosphere can result in beneficial, harmful and non-productive interactions. A proposed new paradigm for studying this interrelationship characterizes these interactions as being *directly* or *indirectly* correlated with the microbial mechanisms affecting seed germination. *Direct* correlations involve a contiguous association between microbes, their mechanisms of action and seed germination. For example, in the case of *direct* correlations, some microbial attribute, whether it is a process or a product, would be independently responsible for changes occurring in the seed or its processes during germination. *Indirect* correlations, on the other hand, involve stepwise, cause and effect responses in the association between seed and microbe during germination. In this case, a microbial process or product would be

instrumental in changing the spermosphere so as to effect other microorganisms or environmental conditions (pH or osmotic potential and nutrient or Oxygen availability) subsequently encouraging an alteration in seed processes. Fox et al. (2003) dissected the topic of causal factors into active and passive roles allowing supplementary development of the intricacies of *direct* correlation. Active roles, usually chemical in nature, cause physiological changes in the seed (i.e. release of growth hormones) that directly affect seed growth (i.e. encourage sprouting) (Fox et al., 2004). Passive roles are physical or chemical in origin and result in morphological changes in the seed (i.e. compromising the integrity of the seed coat) that directly affect other aspects of seed growth (i.e. more rapid exchange of water and oxygen) (Fox et al., 2004).

Beneficial Effects

Beneficial effects of microbes can be defined as being *directly* correlated with seed germination. These effects accelerate normal germination patterns of seeds. The presence of beneficial interactions between microbes and seed germination is illustrated by research performed by Niemi and Haggman (2002) who found that germination of somatic embryos of Scots pine was actively correlated with *Pisolithus* in a contact free culture of the fungus and embryos. They allude to other studies by Niemi et al. in 2000, and 2002, not discussed here, where indoleacetic acid and diamine cadverine, chemicals involving rooting and root growth, are reportedly produced by this strain of fungus possibly making them instrumental in this chemically dictated response. Koaze (1957) also reports an active, beneficial correlation in the production of a chemical present in culture filtrate of *Streptomyces* sp. S-580 functioning as a germination promoter, found to accelerate germination of rice seeds.

Passive associations are reported by a majority of the beneficial studies. Rheeder et al. (1990) report a positive correlation between *Fusarium subglutinans* and undefined 'other' fungi and corn germination in which the mechanisms are unknown. Guttridge et al. (1984) report significant germination increases of seeds inoculated with *Ulocladium charatarum* fungus (74%, 7%) over the control (38%, 2%) after eleven days for two varieties of strawberry. Although chemical influences were not completely ruled out the researchers suggest that infection compromised the seed coat that either removed physical restrictions or chemical inhibitors from the area surrounding the embryo and endosperm. Pfeiffer (1934), a pioneer in this field, discovered that *Symphoricarpose racemosus* seeds would not germinate unless infected by fungi, suggesting that since imbibition occurred, lack of germination was not due to permeability issues, but to mechanical restriction. Further hypotheses conjecture that the fungal growth and subsequent nutrient absorbtion by these microbes broke this mechanical barrier down thus allowing germination (Pfeiffer, 1934). Morpeth and Hall (2000) report significant increases in germination of *Rosa corymbifera* 'Laxa' seed after being enhanced with a commercial compost activator containing a combination of nutrients used to promote microbial activity. Researchers concluded that natural microbial loading was necessary for germination and that the mechanisms involved were mechanical. This is manifest by the splitting of seed coats during enhanced conditions versus intact seed coats of control seed where no microbes were added (Morpeth and Hall, 2000). Control germination yielded 1.8% for non-surface-sterilized/non-inoculated, 0% for surface-sterilized/non-inoculated, and 2.8% for surface-sterilized/inoculated. Compost activator raised the mean percent germination in the non-surface-sterilized/non-inoculated to 52.7%, and the

surface-sterilized/inoculated to 55%. Lastly, Schafer and Kotanen (2004) report enhanced germination of *Solidago nemoralis* and *Verbascum thapsus* in the presence of fungi including *Alternaria*, leaving the mechanisms involved undefined.

Harmful Effects

Harmful effects are defined as having *direct* negative correlations with seed germination. These effects may result when microbes are able to suppress or outgrow defensive mechanisms (Delaney, 1994) or alter normal functions of the host seed. Seven studies were found describing molecular and cytological mechanisms in pathogenesis that *actively* work to chemically alter initial plant morphogenesis and seed germination. Otani et al. (1995) report that there are certain groups of *Alternaria* toxins that have a variety of effects on plant defenses including: AK, AF, and ACT-toxins strongly affect plasma membranes causing invagination, electrolyte loss and precipitation of magnesium and sodium ions; ACR(L)-toxins adversely affect mitochondria and finally AM-toxins work to inhibit CO_2 fixation and cause electrolyte loss. Other literature supports these claims. Kausar and Devi (1987) found that the production of a toxic and mutagenic culture filtrate of *Aspergillus* caused cytological problems in T*rigonella* seeds including anaphase bridges and mis-orientation of the mitotic spindle. Likewise, Zonno and Vurro (2002) indicate that seven of eighteen *Fusarium* toxins belonging to trichothecene A and B groups, known inhibitors of protein synthesis, caused 100% germination inhibition of *Orobanche* seed. Gupta and Chaudhary (1992) report the presence of inhibitory substances in culture filtrate of *Alternaria raphani* that are negatively correlated to the germination of radish seeds. El-Nagerabi and Ahmed (2001) present evidence indicating decreased germination of onion seed upon infestation with *Aspergillus.* In the laboratory,

the Saggai and El-Hilo onion cutivars respectively exhibited an average 20% and 36% drop in germination over the controls 90% and 85%. The researchers failed to define the mechanism involved although they allude to other studies suggesting that toxic metabolites might be involved. Cramer and Lawrence (2003), report increased activation of certain fungal genes in *Alternaria* during infection of *Arabidopsis*, which are designed to provide biochemical protection of fungi against toxic plant compounds thus increasing fungal pathogenicity. Finally, Schafer et al. (1989) describing *Nectria* as producing a detoxifying agent called pisatin demethylase, which works to detoxify phytoalexins, in turn protecting the fungus and increasing its pathogenicity. The researchers (Schafer et al., 1989) also reported enzyme production by *Cocliobolus heterostrophus*, which enhanced its pathogenicity against a pea plant by detoxifying antimicrobial phytoalexins produced by the plant.

Passive, harmful associations are often ambiguous because in most cases the mechanisms of interaction remain undefined. Rheeder et al. (1990), for example, reported a negative correlation between fungal infection by *Diplodia maydis* & *Fusarium* spp. and corn germination. The exact mechanisms are unknown except that *D. maydis* attacks and kills the embryo. Further, Sulaiman and Hussain (1984) report that infection of wheat, rice and barley by *Aspergillus* resulted in decreased germination due to unknown mechanisms. Khanam (1990) reports that germination percentages of wheat were lowered 25% in the laboratory and 24% in the greenhouse setting when compared to the control in the presence of *Drechslera*, *Fusarium*, and *Alternaria* spp. with no defined mechanism of action. Rude et al. (1999) report that two species of *Alternaria* reduced germination of canola by 15% when test infection rates were greater than 20%,

concluding that this infection was caused by the pathogenicity of the species. Mathur and Sehgal (1964) report inhibited germination of Sorghum as compared with the control (92%) in the presence of three fungi, *Rhizopus* (72%), *Alternaria* (80%), and *Aspergillus* (85%); a combinatory inoculum yielded 67% germination. Lastly, Schafer and Kotanen (2004) report a 50% reduction in germination upon infection of numerous plant species with various fungi.

Nonproductive Effects

Nonproductive effects of microbes on seeds may ultimately result in beneficial, harmful, or neutral influences on germination, but these effects are defined as being indirectly correlated with germination because they do not influence the seed directly but alter conditions in the spermosphere. Antagonism or competition between members of the microbial community vying for a place on the seed and for the resources available as a result of seed germination is one illustration of nonproductive effects. Such competition between microbes is described by an experiment performed by Tempe and Limonard (1973) where a sterilized seed is inoculated with *Fusarium* and treated two separate ways. One batch of seed was plated on a nutrient medium and another was planted in non-sterile soil. The plated seed, with no antagonistic presence to Fusarium, yielded 100 % infection while the planted seed remained nearly disease free due to antagonistic soil borne microbes. Lynch (1978), reports an antagonistic interaction between the fungus *Gliocladium* and the bacteria *Azobacter*. In the presence of only the fungus, barley seed germination is decreased to 47%, but upon application of the bacteria, cultured in a nitrogen free media, in consortium with the fungus germination increased to 87%. Although the overall effect was beneficial these interactions did not take place

between the seed and microbes directly and thus are considered non-productive. Lynch (1978) in fact suggests that the balancing synergistic interaction between the fungus and bacteria may have decreased seed germination below its potential due to the depletion of oxygen. Likewise, Khanam (1990) reports that germination percentages of seed treated with *Aspergillus* (96%) increased 6.4% over the control (89.6%) in a greenhouse planting setting, concluding that the effect of *Aspergillus* was antagonistic to other microbes and not directly related to developmental seed processes. In another study (Nelson, 2004) the presence of the bacterium *Enterobacter cloacae* decreased the effects of plant exudates on the growth of *Pythium ultimum,* a fungus, which in turn resulted in decreased disease of germinating seeds.

Other research placed some seed/fungal relationships in a nonproductive category because there were no differences noted over the control or microbial processes lacked the timing for prescribed influences. Fernandez et al. (1994) reported slight influences of germination on Durum wheat when it was contaminated by *Alternaria*. The maximum percent germination difference between uninfected seed and infected seed was 5%. Cromey and Mulholland (1988) conclude that there is no significant germination difference between wheat seed infected with *Alternaria* (98%) and those that were clean (100%). Similarly, Rees et al. (1984) find no significant affect of *Alternaria* infection on wheat seed germination. Moreover Rude et al. (1999) found that *Alternaria alternata* had no affect on germination of canola seeds and thus was considered non-pathogenic. Finally, Zeng et al. (2001) reported the production of inhibitory substances 48 hours after inoculation. Although culture filtrate of A*spergillus japonicus* was negatively correlated with germination of rape, radish and cucumber seed, researchers concluded that

germination would occur before the production of the toxin in-situ and thus have no real affect on the seeds (2001).

Synopsis of Available Research

Analysis of 35 different references, describing interactions between seeds and microorganisms as they are related to germination, assembled from a total of 25 studies supports the segregation of microbial effects on germination into beneficial, harmful and nonproductive associations. It is evident that 20% of the data support beneficial interactions. Fungal mechanisms control five of the seven studies that report beneficial effects, with minimal action from bacteria and unknown microbes. One of the studies, 3% of total references, links the fungus, *Alternaria*, to beneficial effects associated with seed germination. Fungi also control the harmful effects, which account for over half (54%) of the total references. Of the 19 harmful references six are influenced by *Alternaria* and four by *Aspergillus* apportioning these ubiquitous fungi in the harmful category with 29% of the total references. Nonproductive effects account for 26% of the total referenced interactions and are controlled entirely by fungal mechanisms. Over half the nine nonproductive references can be attributed to the fungi *Alternaria* (4) and *Aspergillus* (2), and account for 17% of the total references.

Half of the total references are marked by influences from *Alternaria* and *Aspergillus*. The harmful and non-productive categories are dominated by action from these fungi. *Alternaria* accounts for 32% of the outcomes related to harmful interactions and 44% of those related to nonproductive interactions while *Aspergillus* holds 21% of the harmful interactions and 22% of the nonproductive interactions (Figure 2.1).

Mechanisms of the harmful studies are split between active and passive

associations while those correlated with the beneficial references are biased in favor of passive mechanisms and lingering in active mechanisms. The nonproductive category cannot be separated as to active or passive mechanisms due to the nature of antagonism and the neutral effects present. Of the total references 29% are linked to active associations and 45% are tied to passive associations leaving 25% of references in neutral associations as related to seed-microbe interactions (Figure 2.2).

This analysis shows data supporting beneficial interactions as a minority participant in the seed-microbe interaction. Categorical distinctions in the definition of mechanisms, labeled as active or passive associations, are well defined and seem to support the idea that approximately half of the seed-microbe interactions are due primarily to physical or chemical mechanisms resulting in morphological changes in the seed directly affecting seed growth (passive associations) as opposed to one quarter of these interactions causing physiological changes in the seed by chemical means (active associations) and one quarter having neutral associations.

Natural Resource Management Implications

In conjunction with the Bureau of Land Management, the Forest service is working to bring stability back to the Great Basin, which was disturbed in 1999 by large wildfires. As a result of this disturbance initiatives were developed to help supplement the current, lacking restoration methods. These initiatives were developed in hopes that balance could be restored to this system before invasive species gained complete control of the natural cycles inherent to the survival of the area. One initiative called the Great Basin native plant selection and increase project involved research of the plants native to

the Great Basin ecosystem with the aim of selecting ones suitable for restoration in terms of life history and competitive abilities.

One of issues addressed in this initiative is the characteristic dormancy of certain native species and the possible use of a natural promoter for breaking these dormancy patterns. Acceleration of germination through the breakdown of the seed coat, activation of a physiological trigger or some other means would serve to augment the competitive abilities of hard-seeded species, species prone to the necessities of stratification and other species with inherent dormancy issues by working to counteract the quick germination and establishment of invasive plant species, which frequently hamper re-vegetation of native species in the post disturbance ecosystem.

One specific example of a species involved in this research and being considered for restoration is that of the hard-seeded legume, *Astragalus utahensis*. The genus A*stragalus* is widely distributed throughout the world with $1/6^{th}$ of the total species residing in North America (Rios and Waterman, 1997). The Great Basin houses enough ecological diversity to support various different species of *Astragalus.* Along with the potential for variation comes the likelihood that undesirable species will have space to grow and establish. One problem associated with most *Astragalus* species is their chemical make-up which causes toxicity in livestock and wildlife (Rios and Waterman, 1997) including symptoms such as neurological disturbances, emaciation, habituation, reproductive alterations, and congestive heart failure (James, 1972). Numerous references make mention of *Astragalus cicer* as a non-bloating forage for livestock and wildlife (Acharya et al., 1993; Miklas et al, 1987; Rios and Waterman, 1997; Stout, 1998) inferring that it is toxin free. The Utah native, *A. utahensis,* falls into the same category

as *A. cicer*, and thus is a prospective candidate for use in restoration of disturbed wildlands in the Great Basin. Barneby (Barneby, 1964) described it, common name Utah milkvetch, as a perennial, xerophyte distributed throughout the western United States, northern Mexico and southwestern Canada with an effective elevation ranging from 1250 to 2130 meters. He noted that this tap-rooted, herbaceous plant is found flourishing on calcareous soils located on dry stony hillsides, open gravelly banks and river terraces and is usually associated with sage, oak brush and juniper.

The ecology of *A. utahensis* intersects directly with the arid habitat in which it has evolved especially in terms of seed dormancy (Barneby, 1964; Caudle and Quarterman, 1968). Seed dormancy can be defined as a complex interaction between external environmental and inherent internal germination barriers that must be breached if germination is to occur (Baskin and Baskin, 1998). As described earlier interruption or supplementation of the processes inherent to the cell cycle, by external or internal impetus whether chemical or environmental (Matsushime et al., 1991), is a decisive factor in whether inhibition or acceleration of the germ response occurs.

Arid plants have evolved mechanisms to control germination that can be considered exogenous, described by Nikolaeva (1977) as mechanical and chemical dormancy. These include the presence of chemical inhibitors (Baskin and Quarterman, 1969; Evenari, 1949; Green, 1973; Ziemkiewicz and Cronin, 1981), seed coat impermeability (Caudle, 1968; Evenari, 1962; Miklas et al., 1987; Rolston, 1978; Ziemkiewicz and Cronin, 1981) and, more *Astragalus* specific, the possible presence of a thin, but strong second seed coat (Baskin and Quarterman, 1969). Complimentary studies by Green (1973) and Caudle and Quarterman (1968) acknowledged the elements

of exogenous dormancy when they independently reported substantially low germination rates (9 month time course) in *A. utahensis* seeds (acid treated at 2.8% and non-treated at 0.0%) and *A. tennesseensis* seeds (acid treated at 3.0% and nontreated at 3.0-4.0%). Supplementing the treatments with leaching and removal of the inner seed coat resulted in 92.5% and 100% germination respectively. Miklas et al. (1987) also acknowledged the elements of this dormancy, by describing the hardseeded nature of *Astragalus cicer* and its effect on imbibition and eventual germination. Additional support for the exogenous concepts discussed is relayed by Williams and Elliott (1960) when they insist that survivability, and stand establishment of legumes is tied closely with its seed coat impermeability.

Green (1973) suggests that, in a natural setting, dormancy or germination inhibition is ecologically significant because it delays germination until conditions are right for seedling establishment and growth. This argument is strengthened by the suggestion that dormancy is a critical element in the lifecycle of plants that live in environmentally unstable environments. Dormancy allows the maintenance of an annual seed bank that is relatively flexible in terms of responding in ideal conditions and remaining viable but below the ecological radar during undesirable conditions. Controlling dormancy is critical to the survival of species experiencing extreme environmental conditions. Evenari (1962) describes seed germination in a xeric environment as critical to the life cycle of the plant because the timing, speed, and other intricacies of its germination capacity will determine its survival. Optimal conditions for establishment of a species in extreme environments are outlined by Evenari (1962) when the researcher notes that germination should only take place at a time when moisture

conditions are optimal and should happen quickly. It is also noted (Evenari, 1962) that not all seeds or dispersal units produced in any one year should germinate uniformly, creating a reserve of viable dormant dispersal units in the soil, and that germination should be restricted to habitats that offer the greatest chance for seedling survival. Under natural conditions of moderate competition and less than extreme circumstance these optimum conditions are exhibited by the physiology and life history of *Astragalus utahensis*. The dormancy of this species encompasses nearly all of the elements of this outline proposed by Evenari (1962), in the ideal situation, making it a prime model for the study of arid, hard-seeded species germination.

The viability of the use of this particular plant in restoration projects requires specific attention be given to the nature of its seed dormancy. Researchers have made it abundantly clear that germination of the genus *Astragalus*, in general, is a function of multiple factors (Ziemkiewicz and Cronin, 1981) including temperature, moisture, water potentials, seed source, presence of leachable inhibitory chemicals, and seed hardness coupled with integrity (Baskin and Quarterman, 1969). These dormancy mechanisms stand as prime, inhibitory factors in *Astragalus utahensis* germination. Without physical or chemical triggering mechanisms A. *utahensis* seeds could remain dormant for several years (Baskin and Quarterman, 1969).

While the evolutionary advantages are great in this context, the restorative advantages are nominal. In the context of the germination conditions lined out by Evenari (1962) germination in a restorative context should occur quickly, however, in contrast, it might be beneficial for a plant to germinate relatively uniformly under any conditions of growth, to allow for the development of a population dynamic resistant to

invasive species. Bibbey (1948) points to the germination response of weed seeds as the characteristic, independent of the species competitive abilities and seed production that allows it to maintain viability in the face of disturbances. If in fact there are beneficial effects of micro-organisms on seed germination perhaps these effects could be applied as natural promoters for breaking the seed dormancy of *Astragalus utahensis* and other subject seed. Acceleration of germination in this manner would serve to augment the competitive abilities of these native species by working to counteract the quick germination and establishment of invasive plants,

Competition between plant species for light, water, oxygen, carbon dioxide, and nutrients (Caudle, 1968) creates a dynamic demand for these growth variables. Donald (1963) describes competition as a condition that does not occur unless there is a shortage of one factor that is immediately necessary for the survival of more than one species in an ecosystem. An abundant supply of these materials in a post-fire ecosystem would seem like an ideal non-competitive environment. This profusion of growth factors, however, sets the vital requirement for early establishment of native species. Although *Astragalus utahensis* is able to exist in recently disturbed areas under relatively harsh environmental conditions and has excellent survival characteristics in terms of rapid growth and completion of its life cycle it is unable to compete well with other plants (Green, 1973) thus leaving it at a disadvantage. Life cycles of invasive species, on the other hand, have developed rapid, uniform germination characteristics that are integral to establishment in harsh environments. The critical point in competition for resources arrives quickly creating a hostile environment for native plants unable to acquire sufficient resources for establishment and survival. Successfully competing for factors necessary to plant

establishment and survival is a function of its ability to grow rapidly and complete its life cycle before other plants are able to commandeer these same resources for similar purposes (Caudle, 1968).

As can be seen by this discussion of dormancy and competition *Astragalus utahensis*, and perhaps other hard-seeded species or plants well adapted to harsh environments through other forms of dormancy, remain at a disadvantage in terms of establishment and survival in an environment as raw and open as the post-disturbance ecosystem. A natural promoter of germination as describe by Pfeiffer (1934) and others might alter the subject plants life history characteristics enough to create a competitive advantage for native species. Genetic manipulation, going so far as to remove dormancy by breeding it out of the species, would be difficult because preservation of the elements that make it competitively sound in a post-disturbance establishment regime is critical to its long-term survivability. Use of an impermanent treatment, aiding in a temporary morphological or physiological disruption of exogenous dormancy would necessarily be instrumental in furnishing the species with the necessary competitive edge.

During the course of implementing the native plant selection and increase project researchers at the Shrub Lab run by the Forest Service (USDA), studying the life history and competitive abilities of plants native to the great basin, noticed that fungal contamination of in-vitro germination trials of *Astragalus utahensis*. Researchers noticed that the contamination was resulting in what appeared to be accelerated germination. The two separate fungi isolated from the seed were identified as the ubiquitous genera *Aspergillus* and *Alternaria* of unknown species. It is possible that these fungi could be used in assisting with accelerated germination of these hard-seeds in the field in turn

assisting the case of restoration projects. Future studies will help to not only demonstrate and define the in-vitro and in-vivo effects of fungal infection on seed germination but help establish a foundation for future research into this area with the goal of developing methods for testing these responses with other seeds and the aim of improving restoration efforts by augmenting seed germination such that native species can compete with exotic types.

Conclusion

In a broad sense this review of pertinent literature shows that most microbes affect seed germination negatively due to toxic metabolytes produced as part of pathogenic interactions with the host. It is possible, however, that production of these metabolic products yield positive results because enzymatic digestion works to break down the mechanical barriers that surround seeds, especially relative to hard-seeded species. Given the way in which the subject plant, *Astragalus utahensis*, fits the criterion outlined by researchers discussing the attributes of dormancy in a xeric environment, this plant stands as a prime model for discussion of the interactions of microbes and seeds and their influence on germination.

This review is critical to the development of research pinpointing associations pivotal in beneficial seed-microbe interactions and allows for categorization of future discoveries in this realm of seed science. Specifically, the abundance of data supporting harmful and nonproductive interactions shows a negative trend in the ability of seeds and microbes to optimally coexist in the spermosphere. It is notable, however, that the deficits in data describing beneficial interactions support constructive objectives aimed at the discovery of the presence of these interactions in the spermosphere. The lack of

studies reporting and defining beneficial responses coupled with an awareness of the paradigm provided by this review gives ample rationale for expanding investigations into the reality of active and passive mechanisms as they relate to seed germination. This would facilitate discovery and lead to the definition of definite useful responses that articulate the dynamic nature of the beneficial seed-microbe relationship aimed at developing methods to moderate cases of extreme dormancy that exist in native plants subject to extreme conditions.

Given that the prompt germination of native species in such a competitive domain is crucial to their establishment and survival, the inability of some native species to promptly establish metabolic seed activity, even under optimal conditions, constitutes a formidable barrier to their restorative capacity. Established seed-microbe interactions prompting quicker germination offer a firm basis for analyzing the potential benefit and efficacy of developing these associations as significant dormancy contravention treatments. Accelerating germination with the help of microorganisms would augment the competitive abilities of native species by circumventing prolonged dormancy allowing more competitive responses to conditions where invasive plants previously had the upper hand. Although these methods are not surrogate to molecular attempts at softening dormancy through plant breeding, if viable in the field, such beneficial seed-microbe interactions would give land managers a simple and effective way of rapidly and efficiently restoring native species to disturbed lands.

Literature Cited

Acharya, S.N., Kokko, E.G. and J. Fraser. 1993. Storage duration and freeze-thaw effects on germination and emergence of Cicer Milkvetch seeds. J. Seed Tech. 17: 9-21.

Agrios, G.N. 2005. Parasitism and disease development. In: Plant Pathology. 5th edition. Elsevier Academic Press, New York, pp 77-104.

Barneby, R.C. 1964. Atlas of North American *Astragalus*. Memoirs of the N.Y. Botanical Garden vol. 13 1188 pp.

Baskin, C.C. and J.M. Baskin. 1998. Seeds: ecology, biogeography, and evolution of dormancy and germination. Academic Press. San Diego, CA USA.

Baskin, C.C. and E. Quarterman. 1969. Germination requirements of seeds of *Astragalus tenneseensis*. Bull. Tor. Bot. Club 96: 315-321.

Bibbey, R.O. 1948. Physiological studies of weed seed germination. Plant Physiology 23: 467-484.

Caudle, C. 1968. Studies on the life history and hydro-economy of Astragalus tenneseensis (Leguminosae). Ph.D. dissertation, Vanderbilt University, Nashville, TN.

Caudle, C. and E. Quarterman. 1968. Studies on the life history and hydro-economy of *Astragalus tenneseensis*. Bull. Ecol. Soc. Amer. 49: 108-

Cramer, R.A. and C.B. Lawrence. 2003. Cloning of a gene encoding an *Alternaria* a1 isoallergen differentially expressed by the necrotrophic fungus *Alternaria brassicola* during *Arabidopsis* infection.

Cromey, M.G. and R.I. Mulholland. 1988. Blackpoint of wheat: fungal associations, cultivar susceptibility, and effects on grain weight and germination. New Zealand J. Ag. Res. 31: 51-55.

Delaney, T.P., Uknes, S., Verooij, B., Friedrich, L., Weymann, K., Negrotto, d., Gaffney, T., Gut-Rella, M., Kessman, H., Ward, E., and J. Ryals. 1994. A central role of salicylic acid in plant disease resistance. Science 266(5188): 1247-1250.

Donald, C.M. 1963. Competition among crop and pasture plants. Advances in Ag. 15: 1-118.

El-Nagerabi, S.A.F. and A.H.M. Ahmed. 2001. The effect of black mould (*Aspergillus niger*) on two Sudanese cultivars of onion. Trop. Sci. 41: 95-99.

Evenari, M. 1949. Germination inhibitors. Bot. Rev. 15: 153-194.

Evenari, M. 1962. Plant physiology and arid zone research. Arid Zone Res. 18:175-195.

Fernandez, M.R., Clarke, J.M., DePauw, R.M., Irvine, R.E. and R.E. Knox. 1994. Black point and red smudge in irrigated duram wheat in southern Saskatchewan in 1990-1992. Can. J. of Pathology 16: 221-227.

Fox, S.C., Fernandez, M.R., and R.M. DePauw. 2003. Red smudge infection modifies sprouting response in four wheat lines. Can. J. Plant Sci. 83(1): 163-169.

Green, T.W. 1973. Factors affecting the ecology of *Astragalus libarius* and *Astragalus utahensis* with emphasis on role of insects. Ph.D. dissertation Utah State University. 143 p.

Gupta, D.K. and K.C. Basu Chaudhary. 1992. The effect of culture filtrates of *Alternaria raphani* of radish on seed germination and seedling growth. Intl. J. Trop. Plant Dis. 10(2): 199-202.

Guttridge, C.G., Woodley, S.E., and T. Hunter. 1984. Accelerating strawberry seed germination by fungal infection. Annals of Botany 54(2): 223-230.

James, L.F. 1972. Syndromes of Locoweed poisoning in livestock. Clinical Toxicology 5: 567-573.

Kausar, R. and P. Devi. 1987. Effect of culture filtrate of *Aspergillus niger* on the somatic cells of methi. Indian Bot. Rep. 6(2): 116

Khanam, M. 1990. Seed-borne organisms and effects of different cultures of fungi on germination of wheat varieties. Sarhad J. Ag. 6(4): 407-409.

Koaze, Y. 1957. Germination promotants for plant seed, produced by microorganisms. Bull. of the Ag. Chem. Soc., Japan 22: 91-97.

Lynch, J.M. 1978. Microbial interactions around imbibed seeds. Annals of App. Bio. 89: 165-167.

Mathur, R.L. and S.P. Sehgal. 1964. Fungal mycoflora of jowar (Sorghum vulare), its role in reduced emergence and vigour of seedling and control. Indian Phytopathology 17: 227-229.

Matsushime, H., Roussel, M.F., Ashmun, R.A. and C.J. Sherr. 1991. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 65: 701-713.

Miklas, P.N., Townsend, L.E., and S.L. Ladd. 1987. Seed coat anatomy and the scarification of Cicer Milkvetch seed. Crop Sci. 27: 766-772.

Morpeth, D.R. and A.M. Hall. 2000. Microbial enhancement of seed germination in *Rosa corymbifera* 'Laxa'. Seed Sci. Res. 10: 489-494.

Nelson, E.B. 1990. Exudate molecules initiating fungal responses to seeds and roots. Plant Soil 129: 61-73.

Nelson, E.B. 2004. Microbial dynamics and interactions in the spermosphere. Annual Review of Phytopathology 42: 271-309.

Niemi, K. and H. Haggman. 2002. *Pisolithus tinctorius* promotes germination and forms mycorrhizal structures in Scot's ine somatic embryos in-vitro. Mycorrhiza 12: 263-267.

Nikolaeva, M.G. 1977. Factors controlling the seed dormancy pattern. In: The physiology and biochemistry of deep seed dormancy. A.A. Khan eds. North Holland Publishing Co., New York pp 51-74.

Otani, H., Kohmoto, K., and M. Kodama. 1995. *Alternaria* toxins and their effects on host plants. Can. J. Bot. 73(suppl. 1): S453-S458.

Pfeiffer, N.E. 1934. Morphology of the seed of *Symphoricarpos racemosus* and the relation of fungal invasion of the coat to germinative capacity. Cont. Boyce Thompson Institute 6: 103-122.

Rees, R.G., Martin D.J., and D.P. Law. 1984. Black point in bread wheat: effects on quality and germination, and fungal associations. Australian J. Exp. Ag. and Animal Husbandry 24: 601-605.

Rheeder, J.P., Marasas, W.F.O. and P.S. Vanwyk. 1990. Fungal associations in corn kernels and effect on germination. Phytopathology 80: 131.

Rios, J.L., and P.G. Waterman. 1997. A review of the pharmacology and toxicology of *Astragalus*. Phytotherapy Res. 11: 411-418.

Rolston, M.P. 1978. Water impermeable seed dormancy. Bot. Rev. 44: 365-396.

Rude, S.V., Duczek, L.J. and E. Seidle. 1999. The effect of *Alternaria brassicae*, *Alternaria raphani* and *Alternaria alternata* on seed germination of *Brassica rapa* canola. Seed Sci. Tech. 27(2): 795-798.

Schafer, M. and P.M. Kotanen. 2004. Impacts of naturally occurring soil fungi on seeds of meadow plants. 2004. Plant Ecol. 175: 19-35.

Schafer, W., Straney, D., Liuffetti, L., Van Etlen, H.D. and O.C. Yoder. 1989. One enzyme makes a fungal pathogen, but not a saprophyte, virulent on a new host plant. Science 246(4927): 247-249.

Spohr, A.B., Dam-Mikkelsen, C., Carlsen, M., Nielsen, J., and J. Villadsen. 1998. On-line study of fungal morphology during submerged growth in a small flow-through cell. Biotechnology and Bioengineering 58:541-553.

Stiles, E.I. 1948. Relation of water to the germination of corn and cotton seeds. Plant Physiology 23: 201-222.

Stout, D.G. 1998. Rapid and synchronous germination of Cicer Milkvetch (*Astragalus cicer* L.) seed following diurnal temperature priming. J. Ag. and Crop Sci. 181(4): 263.

Sulaiman, E.D. and S.S. Hussain. 1984. Pathogenicity and effect of germination caused by *Aspergillus* and *Penicillium* species on wheat. Pak. J. of Sci. and Ind. Res. 27: 359-362.

Tempe, J. and T. Limonard. 1973. Seed-fungal-bacterial interactions. Seed Sci. Tech. 1: 203-216.

Vasquez-Ramos, J.M. 2003. The cell cycle and seed germination. Seed Sci. Res. 13(2): 113-130.

Williams, W.A. and J.R. Elliott. 1960. Ecological significance of seed coat permeability to moisture in crimson, subterranean and rose clovers in a Mediterranean-type climate. Ecology 41: 733-742.

Xiong, Y., Zhang, H. and D. Beach. 1992. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 71: 505-514.

Zeng, R.S., Luo, S.M., Shi, M.B., Shi, Y.H., Zeng, Q., Tan, H.F. 2001. Allelopathy of *Aspergillus japonicus* on crops. Agricultural Journal 93(1): 60-64

Ziemkiewicz, P.F. and E.H. Cronin. 1981. Germination of seed of three varieties of Spotted Locoweed. J. Range Man. 34: 94-97.

Zonno, M.C. and M. Vurro. 2002. Inhibition of germination of *Orobanche ramosa* seeds by *Fusarium* toxins. Phytoparasitica 30(5): 519-524.

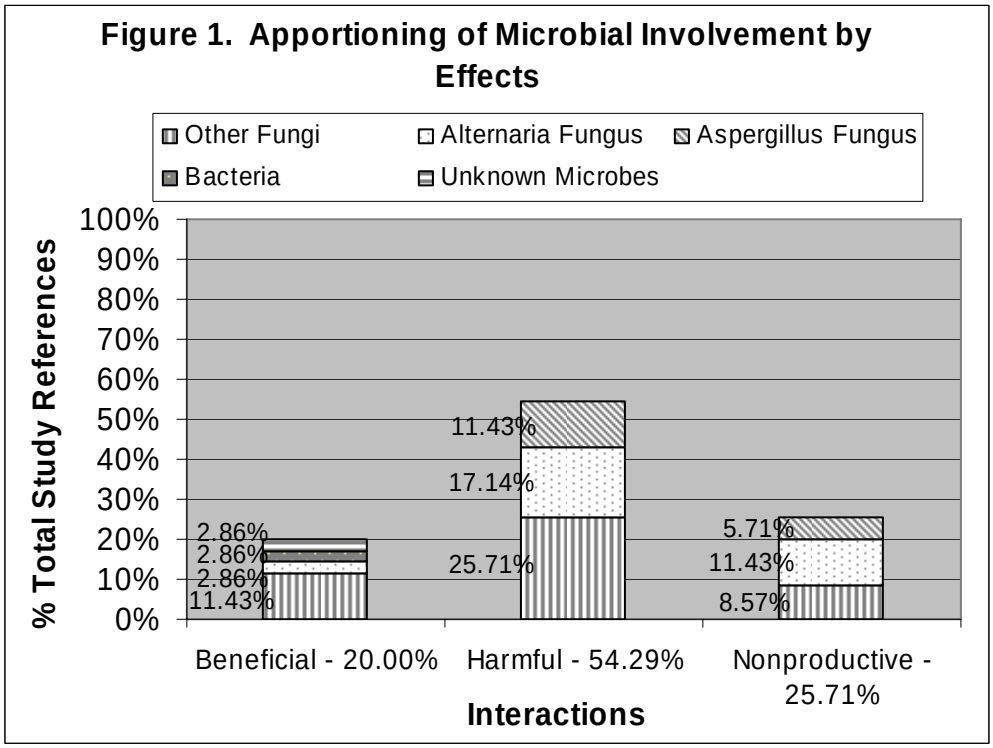

Figure 2.1 – Synopsis of 35 references gathered from 25 studies of microbial effects on seed germination. Beneficial effects account for 20% of the interaction with fungi occupying nearly the whole category. Harmful effects, influenced wholly by fungi, account for about 50% of the interactions. Nonproductive effects occupy 25% of the interactions and are also influenced completely by fungi.

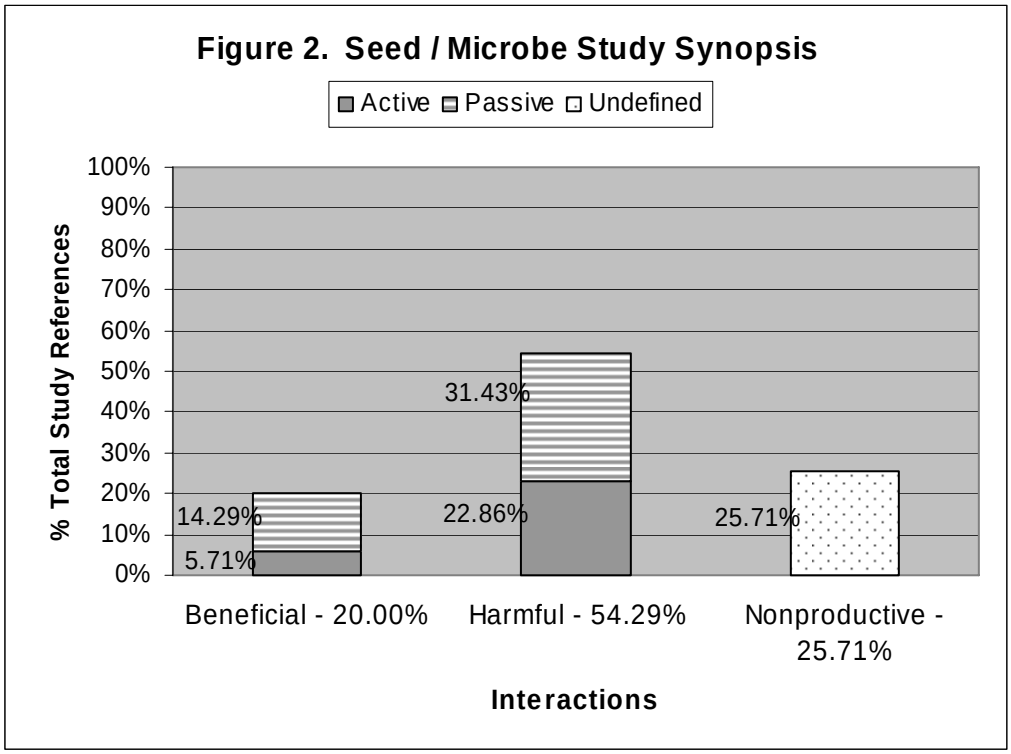

Figure 2.2 – Mechanisms of seed-microbe interactions are apportioned by the way in which the microbes effects germination. Active associations are directly correlated to seed-microbe interactions in that they work by some chemical means to influence the physiology of the seed, enhancing or inhibiting germination. Passive mechanisms can be chemical or physical and work to alter seed morphology thus effecting germination. Non-productive effects result in neither enhanced nor diminished seed germination and often indirectly influence germination in that they affect conditions in the environment surrounding the seed.

APPENDIX A: Mechanisms of Fungal Accelerated Germination of the Hard-Seeded Legume, *Astragalus utahensis* by *Aspergillus* and *Alternaria* Fungi.

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Introduction

In conjunction with the Bureau of Land Management, other federal agencies including the Forest service are working to bring stability back to the Great Basin, which experienced a large disturbance in 1999 caused by wildfires. As a result of this disturbance initiatives were developed to supplement current restoration methods that were aimed at small-scale events and lacked the ability to deal with larges scale disturbances. These steps were taken in hopes that balance could be restored to this system in order to circumvent establishment of invasive species, which have the potential, in a post disturbance environment, to gain complete control of the natural cycles inherent to the survival of the biome. One initiative, the Great Basin native plant selection and increase project, involves developing research on plants native to this biome with the aim of selecting ones suitable for restoration in terms of life history and competitive abilities.

The subject seed whose germination characteristics are addressed as part of this research is that of *Astragalus utahensis*, a hard-seeded legume. The genus A*stragalus* is widely distributed throughout the world with $1/6^{th}$ of the total species residing in North America (Rios and Waterman, 1997). The Great Basin houses enough ecological diversity to support various different species of *Astragalus.* Along with the potential for variation comes the likelihood that undesirable species will have space to grow and establish. One problem associated with most *Astragalus* species is their chemical make-up, which causes toxicity in livestock and wildlife (Rios and Waterman, 1997) including symptoms such as neurological disturbances, emaciation, habituation, reproductive alterations, and congestive heart failure (James, 1972). Numerous

references make mention of *Astragalus cicer* as a non-bloating forage for livestock and wildlife (Acharya et al., 1993; Miklas et al, 1987; Rios and Waterman, 1997; Stout, 1998) inferring that it is toxin free. The Utah native, *A. utahensis,* falls into the same category as *A. cicer*, and thus is a prospective candidate for use in restoration of disturbed wildlands in the Great Basin. Barneby (Barneby, 1964) described it, common name Utah milkvetch, as a perennial, xerophyte distributed throughout the western United States, northern Mexico and southwestern Canada with an effective elevation ranging from 1250 to 2130 meters. He noted that this tap-rooted, herbaceous plant is found flourishing on calcareous soils located on dry stony hillsides, open gravelly banks and river terraces and is usually associated with sage, oak brush and juniper. The ecology of *A. utahensis* intersects directly with the arid habitat in which it has evolved especially in terms of seed dormancy (Barneby, 1964; Caudle and Quarterman, 1968). Seed dormancy can be defined as a complex interaction between external environmental and inherent internal germination barriers that must be breached if germination is to occur (Baskin and Baskin, 1998).

Investigation into the viability of the use of hard seeded species in restoration requires specific attention be given to the nature of their seed dormancy as described. Researchers have made it abundantly clear that germination of the genus *Astragalus* is a function of multiple factors (Ziemkiewicz and Cronin, 1981; Baskin and Quarterman, 1969) including: temperature, moisture, water potential, seed source, presence of leachable inhibitory chemicals, and seed hardness together with integrity. These dormancy mechanisms stand as prime, inhibitory factors in this hard seeded species germination. Without physical or chemical triggering mechanisms seeds could remain

dormant for several years (Baskin and Quarterman, 1969) and while the evolutionary advantages are great in this context, the restorative advantages are nominal.

This research was designed to determine the nature of an accelerated germ response of the hard-seeded legume Astragalus utahensis when inoculated with spores of two separate fungi. Two fungi, isolated from *Astragalus utahensis*, were used as inoculum during the course of this study; each has a ubiquitous distribution (Rotem, 1994). These fungi, *Alternaria* and *Aspergillus*, belong to the Deuteromycetes (imperfect fungi) (Agrios, 2005) rely solely on asexual conidia for reproduction. Both fungi are adaptive to fit any ecological ranging from detritus to the phyloplane (Spooner and Roberts, 2005). Although these fungi belong to the same family they differ in morphology, pathology, and growth requirements.

Rotem (1994) describes *Alternaria* as parasitic or saprophytic. Many factors influence the growth and pathogenicity of this fungus including moisture regime, nutrient availability, and temperature (Agarwal and Sinclair, 1997). Alternatively, *Aspergillus* is described as a saprophyte (Cotty et al., 1994) though it is associated with a variety of plant diseases. The growth of this fungus can be influenced by a number of environmental factors including moisture availability, temperature and other factors like gas composition and pH (Kozakiewicz and Smith, 1994). *Aspergillus* is mesophillic with optimal growth occurring at temperatures between 10 and 40 °C (Kozakiewicz and Smith, 1994) thriving in dry and droughty conditions where lack of moisture is common (Cotty et al., 1994).

Research reports documenting accelerated germination are limited but substantial. Pfeiffer (1934), a pioneer in this field, discovered that *Symphoricarpose racemosus* seeds

would not germinate unless infected by fungi, suggesting that since imbibition occurred, lack of germination was not due to permeability issues, but to mechanical restriction. Further hypotheses conjecture that the fungal growth and subsequent nutrient absorbtion by these microbes broke this mechanical barrier down thus allowing germination (Pfeiffer, 1934). Though many researchers describe similar events and attempt to define the interactions there is limited discussion about tests designed to discover the nature of the observed responses. This research defines the mechanisms involved in an accelerated germination response established in chapter 1 where *in vitro* germination studies confirm beneficial interactions between *Alternaria* and *Aspergillus* fungi and *Astragalus utahensis* seed. Inoculated seed trials (1.0×10^6 spores/mL) exhibited a highly significant difference in percent germination between the uninoculated control at 5.0 % germination and seeds inoculated with *Alternaria* and *Aspergillus* each germinating at 95 % and 55 %, respectively.

This study defines whether the response is related to the physical breakdown of seed integuments or due to some other mechanism.

Materials and Methods

Astragalus utahensis, Utah milkvetch, seed was selected from lot U5-02 to study germination response when inoculated with Aspergillus and Alternaria fungi. Lot U5-02 was collected June 2002 from a site, elevation 1483 m, located on the outskirts of Spanish Fork, Utah at the northeast corner of the intersection of US 89 and US 6 (UTM Zone 12S, E. 449964 N. 4437199). This seed was randomly selected from a relatively uniform population of *A. utahensis* growing on an old river delta in shallow soil.

Fungal cultures of *Aspergillus* and *Alternaria* were isolated from *A. utahensis* seed and used for the production of inoculum to test their influence on germination. *Alternaria* and *Aspergillus,* both species are unknown, were grown and maintained on weak nutrient solution of 1/3 strength potato dextrose agar (1/3 PDA).

The hard-seeded nature of *A. utahensis* required that it be acid scarified (98.0 % H_2SO_4) for up to 20 minutes in order to facilitate imbibition and eventual germination. Scarification was performed in lots of 80 to 150 seeds with up to 8 lots scarified at a time. Placement of seeds in cone sieves, created from polypropylene mesh, facilitated uniform exposure to acid and rapid removal of seed post scarification. After seeds were removed from acid treatment they were immediately rinsed with ddH_2O for 10 seconds in order to neutralize exothermic reactions taking place. A pressurized rinse with distilled water (25 to 30 seconds) completely neutralized the acid ensuring that the seed and its container were clean. Seed was maintained in aseptic conditions in a laminar flow hood until plated or stored to prevent contamination by an outside source.

Inoculation

After acid scarification, the seeds to be used for microscopy were inoculated with a spore solution created from sporulating cultures approximately 1 to 2 months month old. The fungal inoculum was created, adding reverse osmosis water to the petri culture and gently scraping the mycelium to dislodge the spores. One drop of Tween 20 was added to assist in disrupting the hydrophobic barrier between *Aspergillus* spores and H_2O and to make the solutions more uniform in terms of spore suspension. Tween 20 was added to all treatments to ensure consistency between treatment effects. The fungal inoculum was screened through a polypropylene mesh cone to remove agar and mycelial

debris and shaken to disperse spores thoroughly. Spore concentrations were determined by hemacytometer counts. A dilute spore solution (1.00 x 10^6 spores/ mL in H_2O) of both fungi was used for inoculation in the case of precise test. Scarified lots of seed were flood inoculated in cone sieves by recycling spore solutions three times. Use of flood inoculation ensured complete exposure of seeds to the fungal inoculum by momentary suspension of seeds in solution. The thin films of water surrounding the seeds after flood inoculation were critical for spore retention.

Physiological Barrier Tests

The barrier tests were designed to test the response of seeds without the physical presence of the test fungi. Fungal broth cultures made from potato dextrose broth (PDB) were autoclaved and aliqoutted into flasks. PDB was inoculated under aseptic conditions in a laminar flow hood, with 1.0 ml of spore solution (1.0 x 10^6 spores/ml) per 100 ml of culture broth after sufficient cooling. The fungal broth was sealed in autoclave screw cap bottles and incubated over a period of 10 days in the dark at room temperature. Each day a bottle was removed and the filtrate screened to remove large mycelial masses. Filtrate was filtered through a cellulose acetate micro-porous membrane assembly with pores measuring 0.45 μm by means of vacuum filtration to ensure that all fungal parts were separated from the liquid. After that, filtrate was transferred to sterile screw-cap bottles and contained until use.

Seed was plated in varying filtrate regimes over the course of 10 days. Physiological barrier tests were setup in randomized complete block designs replicated 3 times, with two filtrate treatment groups (*Alternaria* and *Aspergillus*) and two control groups (PDB and H_2O) randomized within each of four blocks. Groups of twenty seeds

were randomly selected from treated lots and assigned to each block consisting of a stack of randomized Petri dishes, one for each treatment. Seed was placed on 8.30 cm (diameter) round steel blue seed germination blotters (Anchor Paper Company) wetted with 7.0 ml of either *Aspergillus* or *Alternaria* filtrate or one of the two control treatments, reverse osmosis H_2O or PDB. Seed was placed in the Petri dishes on a polypropylene mesh grid cut to size and dishes were covered and sealed with Parafilm®. Randomized Blocks were lined up and kept in a dark growth chamber at 15 ±2 °C.

Mesh grids were designed to allow for simple exchange of seeds during filtrate exchange in the case of the time course series and the water/chemical soaking experiments. The experimental design allowed a statistical capture of the variation that may have existed as a result of subtle differences in temperature or location in the growth chamber. Seed was exposed to 7.0 ml of filtrate to allow imbibition but prevent over-extension of integuments. Earlier germination trials showed the constant exposure to water caused the seed to imbibe so much water that the integuments would burst and the seed would be allowed to germinate. During the course of the study blotting paper was exchanged to prevent accumulation of chemical concentrations and to allow exposure of seeds to a fresh filtrate also helping to prevent contamination.

Filtrate Regimes

The time course series regime required exposure of seeds to increasingly older culture filtrates. After scarification seed was placed in Petri dishes containing 7.0 ml of filtrate taken from day one incubation. Each day seed was removed from the original petri dish by removing the mesh grid. The excess fluid and used blotter paper was discarded and a 7.0 ml aliquot of Day 2 filtrate was

added to a sterilized round of blotter paper placed in the dish. This process was repeated daily through day 10 allowing the seed involved to gain exposure to a gradual increase of filtrate concentrations. All seed under varying regimes was monitored for germination through day 11.

This filtrate soaking regime required exposure of seeds to Days 2, 4, 6 and 8 culture filtrates for 24 hours prior to plating. Soaking was achieved by placing scarified seed in H_2O, PDB or prospective fungal filtrates for 24 hrs under aseptic conditions. After soaking seed was plated in 5.0 ml aliquots of respective filtrate on steel blue blotting paper. This regime was designed to show an exaggerated response to the respective treatments given prolonged exposure.

The water soaking filtrate regime was designed to test the potential leaching of the seed prior to plating in prospective treatment as described above. During this regime all seed was soaked in reverse osmosis H_2O for 24 hours and then plated in prospective treatments corresponding with filtrates taken from day 2, 4, 6 and 8 fungal culture broth. Germination was monitored up to 11 days

The direct plating filtrate regime involved plating seeds in Days 2, 4, 6 and 8 filtrates immediately after scarification. The direct plating filtrate regime was designed to allow freshly scarified seed to be exposed to filtrate during the critical imbibition time thus enhancing exposure.

Mechanical Barrier Tests

Mechanical barrier tests were designed to test the disruption of the integuments in order to determine impact on seed germination without any fungal influence at all. All seed involved in these tests were scarified and allowed to soak for 2.5 hours in reverse

osmosis water to allow softening of the seed enabling manipulation of the seed coat without effects on the metabolically active portions of the seed. Treated seed was scarified and plated in 5.0 ml H_2O on blotter paper under aseptic conditions.

The first treatment involved removal of the seed coat of acid scarified seed to determine the germination rate under unrestricted conditions and compare that to the previously established germination response. Each seed coat was removed by applying pressure to the outer integuments with tweezers until the integuments burst and the seedling was released onto blotting paper. Care was taken to avoid damaging the cotyledons and any damaged seedlings were removed from the experiment.

The second treatment involved seed coat pricking. A scalpel was used to penetrate the seed coat of acid scarified seed near the hilum in order to create a point of exit for the radical. Care was taken to avoid deep incisions and to ensure appropriate and consistent pricking between seeds. The control for these tests was simply acid scarified seed plated without mechanical disruption of the integuments.

Microscopy

All samples selected for imaging were fixed and dehydrated using similar techniques. Seed was removed from Days 2, 4 and 6, post inoculation, and placed in fixative (2% glutaraldehyde in a 0.06 Molar Na cacodylate buffer) for at least one week or up to 6 months depending on the tests performed as well as the time available for processing. All seed was nicked to ensure proper infiltration of fixatives, dehydrating fluids and resin for LM, as well as intermediate and transitional fluids for SEM. The samples were washed in six 10-minute exchanges of buffer (Na-cacodylate buffer) after which they were rinsed in 6 exchanges of dH_2O for 10 minutes each.

Light and SEM imaging included the optional step of osmium tetra-oxide post-fixation, which assisted with fixation of lipids through cross-linking for LM or helped ensure sample conductivity for SEM.

Dehydration, a crucial step, was performed using a modified version of a method described by Horowitz (1981). This method was employed for the purpose of working with hard seeds because it was absolutely crucial to ensure that all water was removed from the seed prior to resin infiltration and critical point drying. This method differed from traditional methods because it involved extended periods of dehydration (12 to 24 hours) for each ethanol concentration (30, 50, 70, 80, 90, 95%) as well as the 100% Acetone. Variations in time seemed to yield similar results in terms of intact viewing under the scopes as well as proper resin infiltration and complete critical point drying. Traditional methods require brief dehydration periods of 10 minutes per ethanol concentration as well as brief periods allotted for resin infiltration. The major concern with these shortened time periods arose from incomplete infiltration of resin, which indicated possible presence of water in the sample resulting in incomplete dehydration or improper infiltration. The lengthier method was adapted for safety to ensure that once seeds had undergone the lengthy, resource consuming, processing step they were ready for critical point drying or embedding.

Samples undergoing processing for LM were embedded in low viscosity Spurr's resin. After samples were trimmed and sectioned using an ultra microtome sections were placed on slides and viewed under a light microscope. SEM preparation included further processing to remove all excess water and acetone to prepare seed for exposure to conditions present in a vacuum-sealed space. Critical point drying with acetone as the

intermediate fluid and CO_2 as the transitional fluid was performed in five 15 minute CO_2 soaking periods interrupted by 5 minute drain/vent period to allow excess Acetone to be removed and to cool the chamber and exchanged the CO_2.. Finally, the specimens are mounted and sputter coated with gold for 3 minutes. All samples were viewed on the JOEL 840 at 12kV under varying conditions of magnification, working distance, spot size and aperture with minimal charging.

Experimental Design

All data involving germination over time were analyzed using Poisson regression. Significance of the terms is determined by the probability that the difference between mean counts of each coefficient compared to the intercept is zero (null hypothesis). The Chi square values produced by the model are a measure of how close the means come to proving the null hypothesis. A large Chi square value indicates greater probability that the mean of the control (intercept) is different from the treatments (coefficients). A probability value (p value) is calculated from the relationship between control and treatment values projecting the degree of difference between the means. An alpha level of 0.05 is used to determine statistical significance with a p value of < 0.05 labeled significant.

The experimental design, a randomized complete block design, allowed statistical capture of the variation that existed as a result of subtle differences in temperature, location and other variable within a test location, environment or site. Analyses showed significance in the independent variables (i.e. block and test.) in all experiments with the exception of the direct plate and time course series tests. Blocks were insignificant in the direct plate test while tests were insignificant in the time course series; all insignificant

independent variables were left out of the regression model. The interactions between other significant independent variables were highly significant indicating their usefulness and necessity in describing the variability that existed among treatments. In the case that statistical differences ($p < 0.05$) existed between treatments it is essential to express those differences as a function of the significant independent variables like block, test, site and year, keeping them in the model. In the case of the data described in this paper the intercept (PDB control) is the base value to which all other treatments are compared.

Random assignment of seed to their prospective treatments allows causal inferences to be made. Responses identified as statistically significant are implicit in the hypothesis that fungal filtrate has an accelerating effect on the germination of this hard-seeded species. Although inferences to the general population of *A. utahensis* plants throughout their native range cannot be made, there is a definite confirmed response for the seed lot used in this study gathered from the Spanish Fork, Utah site.

Results

The response established *in vitro* showed called the precise spore count germination tests indicated a significant accelerated germ response when seed was treated with *Alternaria* and *Aspergillus* fungi, 75.0 % and 45.0 % germination, respectively. (Figure A.1). Analysis of seed coat treatments (Figure A.1) showed a definite significant response ($P < 0.0001$) between the mechanically treated seed and the scarified control germinating at 15.0 %. The seed coat removal yielded 100.0 % germination while the seed coat pricking showed 25% germination. *Alternaria* treated seed showed no significant difference (0.0 %, $P=0.4817$) from the PDB control (0.0 %) during the Day 4 direct plate test. The time course series indicated significant differences ($P < 0.0001$),

though impractical in terms of the responses exhibited *in-vitro* and during the mechanical barrier tests (> 50.0 5 germination), between the *Alternaria* (10.0 % germination) and the PDB control (5.0 % germination) (Figure A.2).

The scarified control treated by soaking in water yielded similar results (80 to 85 % germination, $P < 0.0001$) in both the filtrate and water soaking tests (Figure A.2). *Aspergillus* treated seed showed a highly significant ($P < 0.0001$) response that was consistent during the Day 4 direct plate and time course series filtrate tests. Both tests yielded 10.0 % germination over the PDB control (0.0 %) (Figure A.2). *Aspergillus* Day 8 direct plate treatments show a highly significant response (30.0 %, $P < 0.0001$) over the PDB control (0.0 %) (Figure A.3). Overall the filtrate soaking test as well as the water soaking test yielded higher germination in all cases including the treatments and controls (Figure A.2) with *Alternaria* germinating at 10.0 % and 30.0 % germination and *Aspergillus* germinating at 20.0 % and 55.0 %, for the respective tests. It is notable however, that the treatment values did not exceed the PDB control (25.0 % and 55.0 %, for the respective tests), which would indicate no real valid significant difference between germination, values in the filtrate and water soaking tests (Figure A.2).

Discussion

In an undisturbed environment, seed dormancy is ecologically significant because it delays germination until conditions are right for the establishment of seedlings (Green, 1973). This argument is strengthened when Green (1973) suggests that dormancy is critical to the lifecycle of plants living in environmentally unstable conditions because it allows for the maintenance of an annual seed bank. This germ bank allows for relatively keen flexibility in terms of rapid response in ideal conditions and continued viability

during perilous seasons. In short, dormancy or control of seed germination is critical to the survival of species under certain undesirable environmental conditions.

Dormancy is a complex interaction between external environmental and inherent internal germination barriers that must be breached if germination is to occur (Baskin and Baskin, 1998). This inability of a seed to resume metabolic processes despite the source of inhibition is directly linked to reactions taking place at a cellular level. Growth of a seedling made possible by completion of the four phases of the cell cycle (G1, S, G2 and mitosis) relies on a complex series of responses including DNA replication, protein synthesis, and positive or negative feedback inhibitions (Vasquez-Ramos, 2003). Interruption or supplementation of these processes by external or internal impetus, whether chemical or environmental (Matsushime et al., 1991), at the gate of the cycle (Xiong et al., 1992), decides whether inhibition or acceleration of the germination response occurs. Managing the cell cycle at its gate by considering the presence or absence of these stimuli is the key to understanding seed dormancy and germination.

Nikolaeva (1977) made two solid distinctions within the context of organic dormancy, or inherent internal seed germination barriers, grouping germination according to the barrier mechanisms involved, endogenous or exogenous dormancy. Endogenous dormancy is related to the physiology of the seed and its developmental characteristics, while, exogenous dormancy is related to the presence of chemical inhibitors, impermeable seed coats and growth restricting structures; the latter is one of the instrumental factors in seed-microbe interactions. Complimentary studies by Green (1973) and Caudle and Quarterman (1968) acknowledged the elements of exogenous dormancy when they independently reported substantially low germination rates, over a

9-month time course study in *Astragalus utahensis* and *A. tennesseensis* seeds. Miklas et al. (1987) also acknowledged the elements of this dormancy, by describing the hardseeded nature of *A. cicer* and its effect on imbibition and eventual germination. Williams and Elliot (1960) provide additional support for the exogenous concepts discussed when they insisted that survivability, and stand establishment of legumes is tied closely with seed coat impermeability.

The influence of microorganisms on germinating seeds has been studied on a limited basis. One of the earliest studies dealing with this phenomenon published by Pfeiffer (1934) suggested that fungi are essential for the germination of *Symphoricarpos racemosus* seeds because the microorganism compromised seed coat allowing emergence of the radical. In a more recent study Nelson (2004) suggests that seeds have evolved in close association with a very diverse population of microbes in the environment surrounding the seed, also known as the spermosphere. Nelson (2004) attributes the proposed relationship, in part, to the conditions perpetuated as a seed begins its metabolic processes. One major component of this dynamic interaction of microbes in the spermosphere is the presence of seed exudates (Nelson, 2004). These soluble carbon sources (Lynch, 1978) are composed of natural cell components including sugars, amino acids, and proteins, which serve as stimulants for microbial growth (Nelson, 1990). They encourage the microbes to arrest their natural state of dormancy, caused by low nutrient availability, and begin growth and activity (Lynch, 1978; Nelson, 1990; Nelson, 2004).

In context of this relationship within the spermosphere there are multiple outcomes of increased microbial activity. Microbes are capable of beneficial, harmful or nonproductive effects on seeds. These effects can be characterized as directly or

indirectly correlated with the mechanisms affecting seed germination. Direct correlations involve a strong connection between the microbe, the mechanism of action and seed germination. For example, in the case of direct correlations, some fungal aspect, whether it is a process or a product, would be solely responsible for changes occurring in the seed. Indirect correlations, on the other hand, involve stepwise cause/effect reactions in the fungal/seed relationship. In this case a fungal process or product is responsible for a change in the spermosphere, in turn affecting other microorganisms or environmental conditions which subsequently encourage an alteration in seed dormancy and germination processes.

The key in defining the mechanisms of action as mechanical or physiological was to eliminate the physical presence of fungi in a set of treatment regimes. Exposure of seed to fungal products absent of any actual fungal parts allows clear separation of these mechanisms. The established *in vitro* response included the presence of the test fungi through direct inoculation of seed with respective spore solutions. Filtrate tests, in which PDB was used as a fungal growth medium, required the removal of fungal parts through filtration. The 0.45µm pore size used prevented any fungal parts from passing through and contaminating the sterile filtrate. Such a small pore size however was prone to blockage because the fungal parts often prevented the flow of filtrate through the assembly requiring that the filter to be changed.

The design of the time course experiment allowed an element of time to be incorporated in that the seed was exposed to culture filtrate that had been incubating for incrementally increasing periods of time. This was designed to imitate the exposure, a

seed might experience during the course of a regular inoculation, to chemicals or metabolites produced by the fungi over time

The direct plate regime was designed to test exposure of seeds to concentrated lots of filtrate without the element of soaking that existed in the filtrate soaking regime. Throughout the multiple seed scarifications performed it was observed that seed imbibed fluids immediately after scarification. Exposure of freshly scarified seed to filtrate would allow immediate imbibition of any active elements of the filtrate before imbibition of seed by exposure to rinse water or soaking treatments. This design would allow for immediate determination as to whether the filtrate effects germination in a more direct and measured way. This test was also designed to bolster data from the Time course series by allowing direct determination of the effects of immediate exposure and imbibition of filtrates. It can be noted that *Aspergillus* treated seed showed significant increases in germination over both the PDB and water controls during the direct plating. These values are statistically significant ($P < 0.0001$) for all Days except Day 2 showing an increase in the potential for effects by *Aspergillus* filtrate over time. The time course series also showed a significant increase over the control values indicating that there is an element of practical significance that can be attributed to the physiological effects of *Aspergillus* treated seed *in* vitro. Another indication of this practical significance is the similarity between *in vitro Aspergillus* treatment responses (45.0 % germination) and Day 8 direct plate treatments (30.0 %). These similarities show that physiological effects mandated by Day 8 filtrate closely mimic those experienced by *in vitro* germination trials tracked through Day 11.

The filtrate soaking regime was designed to test an exaggerated response of seed to filtrate to determine if there was a definite chemical response affecting the physiological germination characteristics of the seed.

The water soaking filtrate regime was designed to test the presence of a leachable inhibitory substance and to test the response of seed to the culture filtrates and control treatments after the potential inhibitor had been leached. As suggested in the introduction *A. utahensis* might well have some dormancy issues associated with a leachable inhibitory substance. It is true that a lengthy soak yields higher germination rates in water soaked seed over the control and there is an dark brown to amber colored leachate that appears in the soaking bath however, the presence of an inhibitory substance is elusive. Field tests reported in Chapter 1, where water soaked seed was planted along side control seed yielded results that showed the control seed out performed the water soaked seed. There are multiple factors that may have determined this response including seed dry down and refrigeration prior to planting each having a potential effect on seed viability. Field conditions may have also favored control seed over water soaked seed. Another factor may have been the propensity of imbibing seeds to rupture their integuments if they are suspended in liquid for long periods of time. Some preliminary germination tests showed that attempted germination of control and treated seed in a volume of liquid large enough to suspend the seed was observed to be linked to premature rupture of the seed coat. If in fact most seed were already awake in terms of their metabolic processes it is possible that the seed could have died after dry down and exposure to harsh winter conditions or could have been induced to germinate at the

slightest sign of moisture or warmer temperatures in the fall months and again perished in during the harsh winter months.

Identical responses by the scarified control in these filtrate and water soaking tests show that increases in germination yielded by treated seed from the filtrate soaking to the water soaking tests was due primarily to effects of water soaking. The filtrate tests show some response for *Alternaria* but nothing near the acceleration exhibited by the fungal inoculum and mechanical tests. The time course series and the direct plating test, those that close mimic conditions of exposure present during actual inoculation trials show 5.0 % germination at most indicating that the response exhibited *in vitro* under inoculum conditions is not related to any physiological changes experienced by the seed. Though the water soaking treatment indicated substantial germination for *Alternaria* (30.0 %) and *Aspergillus* (55.0 %), the control treatments experienced an increase in germination as well.

It was assumed that soaking for a long period of time would allow stronger determination of the presence of active compounds in respective treatments. The water control allowed detection of the osmotic affects of the Pure Broth to be detected. It was evident that Alternaria acted more like the pure broth treatment yielding little to no germination response. Aspergillus did however yield a measured response with the water control having the greatest response. It is possible that osmotic potential played a large part of the seed responses because the seeds that were treated with water exhibited a higher germination response than those involving the broth treatments. Seed treated with Aspergillus filtrate experienced a slightly elevated response compared to the pure broth and the Alternaria filtrate treatments. This may have been due to an adjustment of the

osmotic potential by Aspergillus because of greater metabolism of the broth. This decrease in osmotic potential would have allowed the seed to imbibe more water and leach possible inhibitory substances from the seed in greater quantities similar to the water control seed.

There was no definite observation that the seeds exposed to any of the treatments were more prone to differing germination responses due to the prolonged soaking. In fact all the soaking treatments yielded a portion of seeds that had burst or begun to germinate before initial plating occurred. These seeds were eliminated from the tests to prevent interference in the data that was to be gathered. These tested are inconclusive as to the origin of germination patterns except in the element that the Aspergillus treatment acted similarly in the time course series (where the element of soaking was absent) and in the filtrate soaking (where the element of soaking was present). This alone serves to add validity to the *Aspergillus* filtrate response being linked to a physiological mechanistic effect. The lack of response by *Alternaria* also tells a story. Essentially when *Alternaria* is absent there is no acceleration of germination. This would indicate that *Alternaria* works mostly by mechanical means in its affects of an accelerated germ response.

Mechanical barrier tests were designed to show how significantly seed integuments impede germination of the *A. utahensis* seed. This test was the mechanical counterpart to the physiological filtrate tests in that there were no fungi present. This attempt to correlate a mechanical breach of the integuments with germination as a result of fungal inoculation at least suggests that the barrier that exists due to the integuments is one of the prohibitive factors in *A. utahensis* germination. The seed coat removal test showed that nearly all seeds germinated when the barrier was removed. This doesn't

pinpoint mechanical restriction as the single element in this determination, where an inhibitor is also a likely possibility, but rapid and nearly complete germination of seed undergoing this treatment suggests that mechanical restriction is a pivotal point in the study of these processes.

It is difficult to separate the confounding effects of restrictive integuments and an inhibitive leachate because in order to remove the seed coat the seed had to be allowed to imbibe which required soaking. This, in turn, implies that some leaching occurred. The treatment involving seed coat pricking was aimed at providing a minimal exit point for the radical in order to determine if this was sufficient to promote high germination rates. This method roughly represents a scaled down to version of fungal penetration where the fungal mycelium metabolize portions of the integuments thus weakening their integrity. This single rupture point, as it were, would serve to show how the seed might react to a small penetration into the seed coat and whether or not significant germination might occur as a result. The small scalpel puncture made near the hilum yielded some results that significantly greater than the control but there were some noticeable difficulties associated with this treatment. Most radicals that protruded from this puncture were malformed and significant damage was observed. It is evident that puncture of the seed in this vital area effected normal development of the radical. It is likely that the radical was punctured or cut upon application of this treatment resulting in deformed growth patterns in the radical.

Comparison of the precise fungal involved tests and the mechanical barrier tests show that the response of *Alternaria* is similar across the board. Both tests show

accelerated germination with *Alternaria* germinating at 75.0 % for the precise fungal inoculum test and 100.0 % for the seed coat removal tests.

Microscopy was used to detect interactions at the surface of the integuments as well as within the seed. Light microscopy was useful in detecting the relationship between treatment and expansion of the cotyledons. Seed extracted from in-vitro germination trial where an accelerated germ response was established (Chapter 1) and observed in a post-preparative state exhibited differences in the size and orientation of cotyledons. Seed treated with both *Aspergillus* and *Alternaria* showed greater signs of swelling and expansion in the cotyledons while the cotyledons in control seed remained compact (see Image A.1-A.3).

More general observations were also made under the light microscope including observations of seed in pre-treatment conditions. These observations served as a baseline for the interpretation of SEM images. The crucial observations occurred in the realm of the control seed. It was noted under a light microscope that the integuments of scarified control seed were entirely intact showing superficial wound craters created by the scarification process (Image A.4). *Alternaria* treated seed exhibited highly disrupted integuments when compared with control seed (Image A.5). The cracks observed in the integuments of *Alternaria* treated seed were similar to those observed under the SEM (Image A6). It is notable that both images show fungi bridging the gaps in the coat. It is likely that if this cracking was not related to fungal growth and disposition and merely a function of the preparative processes inherent to electron microscopy (Image A.7) the fungal hyphae would have shown signs of stress and likely tearing across the gaps. Many of the cracks in the seed coat do not exhibit the local fracturing clearly observed in SEM

images. These other fractures exhibited a more longitudinal splitting effect implying that some force was involved in stretching the seed coat. Since this type of fracturing was not observed in control seed (Image A.4) that had not been processed for electron microscopy it is likely that the stretching is due to expansion of the cotyledons in the presence of fungal metabolism of the integuments. It is interesting to note however that the stretching that occurred in unprocessed treated seed was observed in processed control and treated seed. It is evident from these observations that the integuments undergo some alterations during processing. It is notable that *Aspergillus* treated seed exhibited minor effects on the integuments under light or SEM imaging that may be due to stress or some other indistinguishable effects (see Images A.8 and A.9).

Data from the physiological barrier tests indicate that *Alternaria* treated seed treated in the physical absence of the fungus yield relatively little germination with the highest germination, 10.0 %, exhibited in the time course series. This indicates that the effects are related in part to the chemicals produced by the fungus during growth which assist in breaking the mechanical seed barriers. LM and SEM observations indicate a definite coupling effect of mechanical breakdown. Comparison of the established inoculation tests discussed in chapter 1 with the mechanical barrier tests shows that the responses exhibited are comparable to each other. When these data and observations are taken together it is evident that the accelerated germination experienced by *Alternaria* treated seed is mostly a function of compromise of integuments through their physical and chemical breakdown by fungal mechanisms.

Observations through SEM and light microscopy support the idea that the *Alternaria* treatment assists accelerates germination in this hard-seeded legume by

physically weakening the integuments. Imaging indicated that this relationship allowed the cotyledons to expand (see Image A.1 and A.2) which in turn would help the radical to penetrate the seed coat. It is probable that this softening effect assists the seedling in shedding the integuments during emergence allowing the cotyledons to begin photosynthesis providing the energy necessary for establishment. The mechanisms of accelerated germination as influenced by *Aspergillus* fungi are more elusive. The fungal response is slower than *Alternaria* showing that there may be less physical elements involved in the process.

Light microscopy was also used to observe sectioned seed. Some distinct fungal elements were observed including penetration of hyphae into the seed coat as well as extreme digestion of integuments and cotyledons with little distinction. Overall, attempts to visualized fungal growth and development on the seed surface were most successful when SEM was employed

Evidence gathered from SEM images showed rapid growth including infection and colonization processes early on with Day 2 *Alternaria* images (Image A6) indicating that the fungus was quick to establish and efficient in metabolizing seed tissue. *Aspergillus* images, on the other hand, showed slow development of efforts to infect and colonize with Day 4 images yielding limited results (Image A9)

As can be concluded from the previous discussion, there are numerous ways that seed germination can be affected through microbial action. The definitions of some of these factors will add to the general body of scientific knowledge regarding the intricacies of accelerated seed germination in response to microbial action.

Literature Cited

Acharya, S.N., Kokko, E.G. and J. Fraser. 1993. Storage duration and freeze-thaw effects on germination and emergence of Cicer Milkvetch seeds. J. Seed Tech. 17: 9-21.

Agarwal, V.K. and J.B. Sinclair. 1997. Principles of seed pathology. 2nd eds. CRC Press. Boca Raton, Florida.

Agrios, G.N. 2005. Parasitism and disease development. In: Plant Pathology. 5th edition. Elsevier Academic Press, New York, pp 77-104.

Barneby, R.C. 1964. Atlas of North American *Astragalus*. Memoirs of the N.Y. Botanical Garden vol. 13 1188 pp.

Baskin, C.C. and J.M. Baskin. 1998. Seeds: ecology, biogeography, and evolution of dormancy and germination. Academic Press. San Diego, CA USA.

Baskin, C.C. and E. Quarterman. 1969. Germination requirements of seeds of *Astragalus tenneseensis*. Bull. Tor. Bot. Club 96: 315-321.

Caudle, C. and E. Quarterman. 1968. Studies on the life history and hydro-economy of *Astragalus tenneseensis*. Bull. Ecol. Soc. Amer. 49: 108-

Cotty, P.J., Bayman, P., Egel, D.S. and K.S. Elias. 1994. Agriculture, aflatoxins, and *Aspergillus*. In: The genus Aspergillus from taxonomy and genetics to industrial application. Powell, K.A., Renwick, A. and J.F. Peberdy eds. Plenum Press, NewYork p 1-27.

Green, T.W. 1973. Factors affecting the ecology of *Astragalus libarius* and *Astragalus utahensis* with emphasis on role of insects. Ph.D. dissertation Utah State University. 143 p.

Horowitz, J. 1981. A new preparative technique for studying dry seeds of pisum sativum with the aid of transmission electron microscopy. Micron 12:139-146.

James, L.F. 1972. Syndromes of Locoweed poisoning in livestock. Clinical Toxicology 5: 567-573.

Kozakiewicz, Z. and D. Smith. 1994. Physiology of Aspergillus. In: Biotechnology Handbooks 7 Aspergillus. J.E. Smith eds. Plenum Press, New York. P 23.

Lynch, J.M. 1978. Microbial interactions around imbibed seeds. Annals of App. Bio. 89: 165-167.

Matsushime, H., Roussel, M.F., Ashmun, R.A. and C.J. Sherr. 1991. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 65: 701-713.

Miklas, P.N., Townsend, L.E., and S.L. Ladd. 1987. Seed coat anatomy and the scarification of Cicer Milkvetch seed. Crop Sci. 27: 766-772.

Nelson, E.B. 1990. Exudate molecules initiating fungal responses to seeds and roots. Plant Soil 129: 61-73.

Nelson, E.B. 2004. Microbial dynamics and interactions in the spermosphere. Annual Review of Phytopathology 42: 271-309.

Nikolaeva, M.G. 1977. Factors controlling the seed dormancy pattern. In: The physiology and biochemistry of deep seed dormancy. A.A. Khan eds. North Holland Publishing Co., New York pp 51-74.

Pfeiffer, N.E. 1934. Morphology of the seed of *Symphoricarpos racemosus* and the relation of fungal invasion of the coat to germinative capacity. Cont. Boyce Thompson Institute 6: 103-122.

Rios, J.L., and P.G. Waterman. 1997. A review of the pharmacology and toxicology of *Astragalus*. Phytotherapy Res. 11: 411-418.

Rotem, J. 1994. The genus *Alternaria*. The American Phytopathological Society, St. Paul Minnesota USA.

Spooner, B. and P. Roberts. 2005. Fungi. Harper Collins Publishers, London.

Stout, D.G. 1998. Rapid and synchronous germination of Cicer Milkvetch (*Astragalus cicer* L.) seed following diurnal temperature priming. J. Ag. and Crop Sci. 181(4): 263.

Vasquez-Ramos, J.M. 2003. The cell cycle and seed germination. Seed Sci. Res. 13(2): 113-130.

Williams, W.A. and J.R. Elliott. 1960. Ecological significance of seed coat permeability to moisture in crimson, subterranean and rose clovers in a Mediterranean-type climate. Ecology 41: 733-742.

Xiong, Y., Zhang, H. and D. Beach. 1992. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 71: 505-514.

Ziemkiewicz, P.F. and E.H. Cronin. 1981. Germination of seed of three varieties of Spotted Locoweed. J. Range Man. 34: 94-97.4. Horowitz, J. 1981. A new preparative technique for studying dry seeds of pisum sativum with the aid of transmission electron microscopy. Micron 12:139-146.

Figures

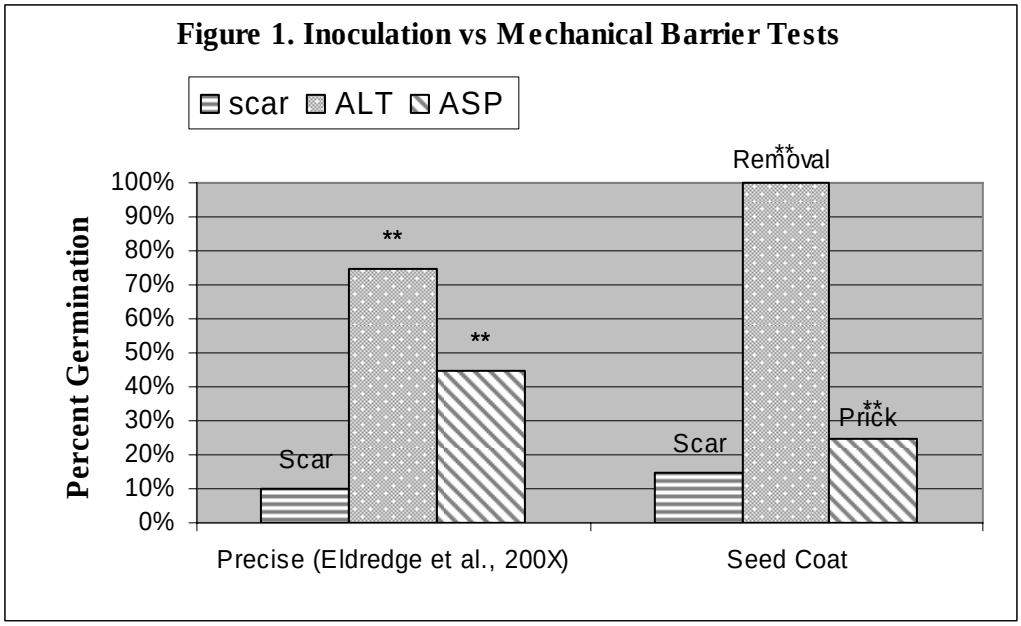


Figure A.1: Comparing the established inoculation tests and the mechanical barrier tests. **P value < 0.0001

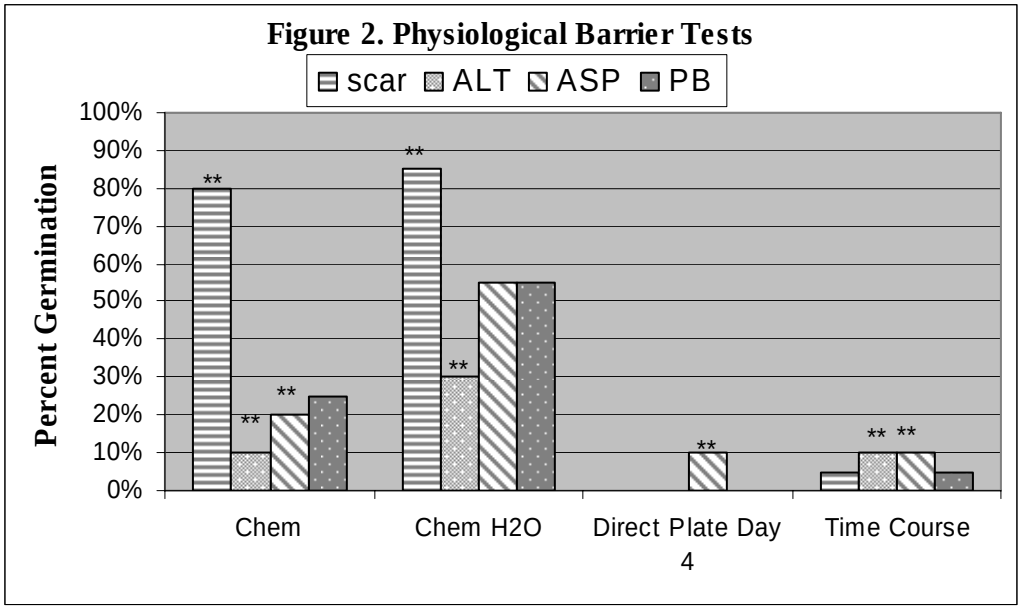


Figure A.2: Filtrate tests to allow determination of the physiological effects on seeds.
** P value < 0.0001

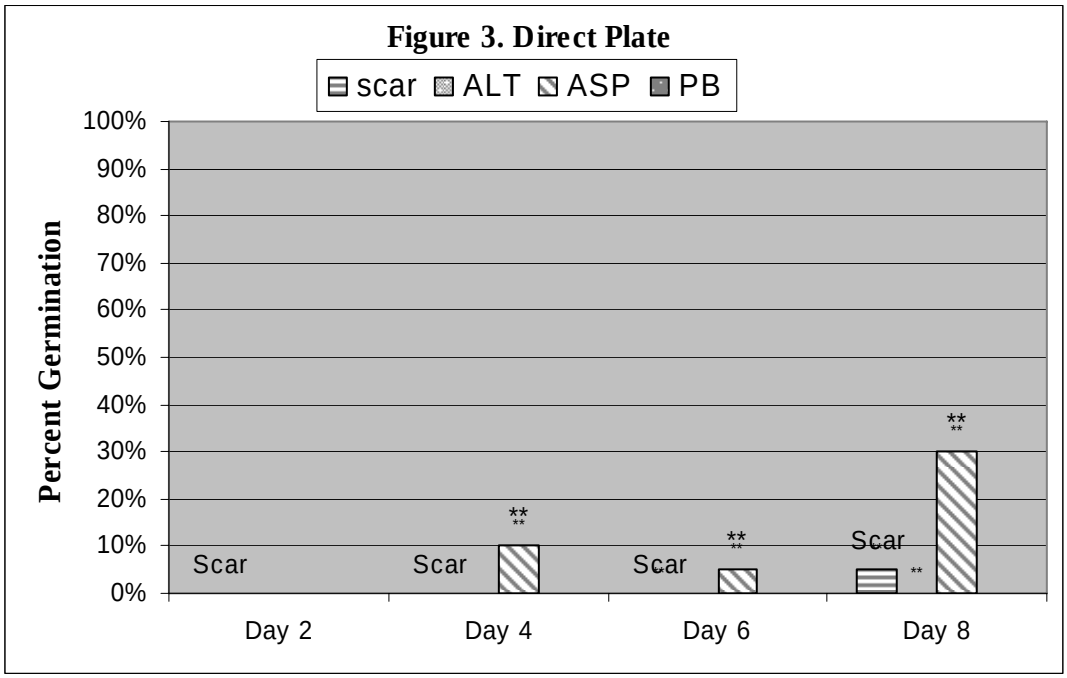


Figure A.3: Direct plate tests designed to test imbibition and physiological response of seeds to filtrate immediately post scarification.
** P value < 0.0001

Images

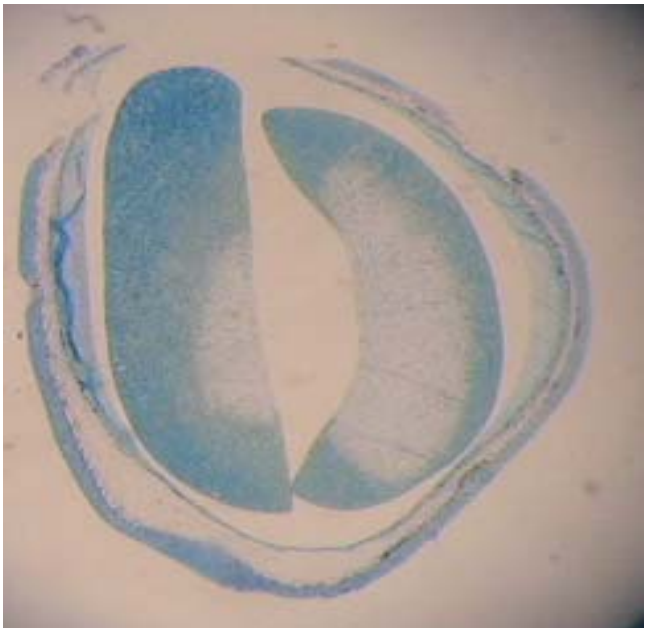


Image A.1: *Aspergillus* Day 3 Cotyledon Swelling

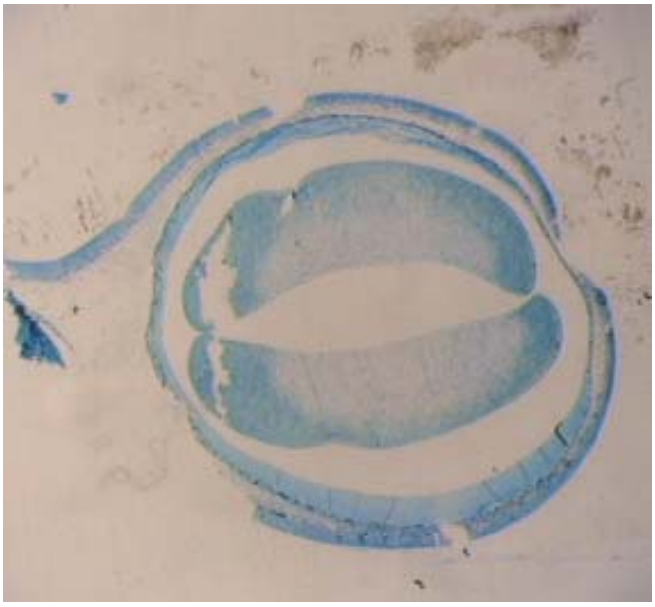


Image A.2: *Alternaria* Day 3 Cotyledon Swelling

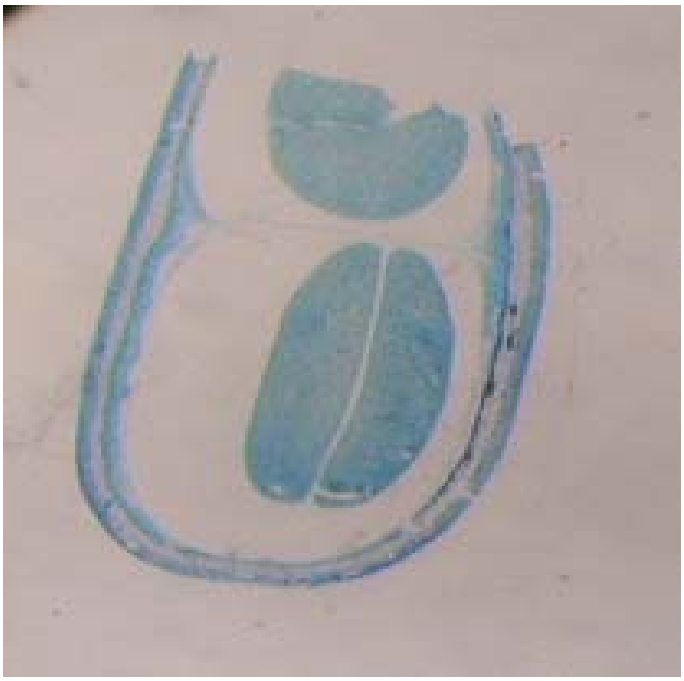


Image A.3: Control Day 3 Cotyledons not Swelling

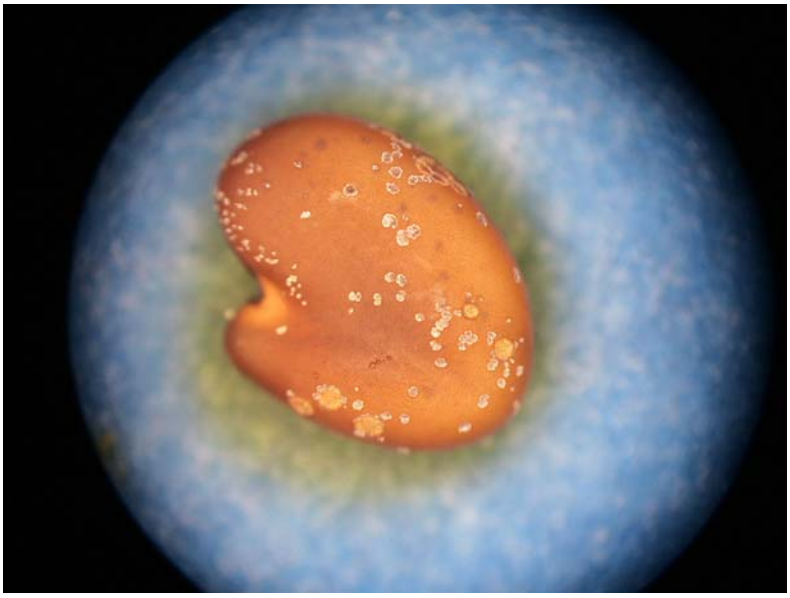


Image A.4: Control seed under LM exhibiting no cracking.

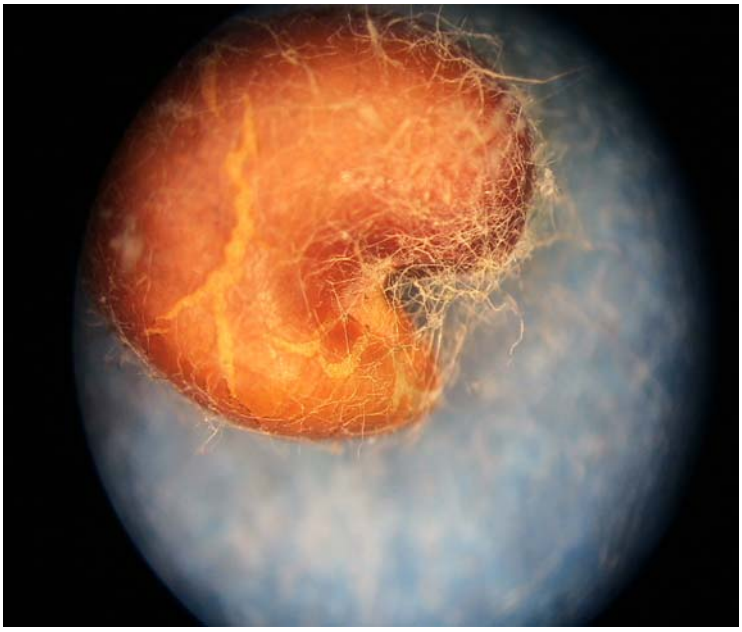
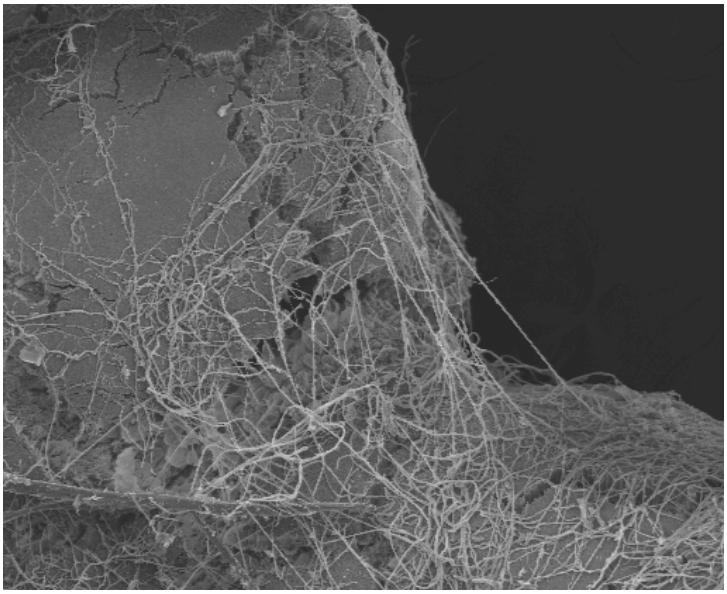


Image A.5: *Alternaria* treated seed under LM seed exhibiting cracking and hyphal elements.



Alt D4: 12kV 100X Spot 9 WD 25 Ap 3

Image A.6: *Alternaria* treated seed under SEM exhibiting cracking and hyphal elements.

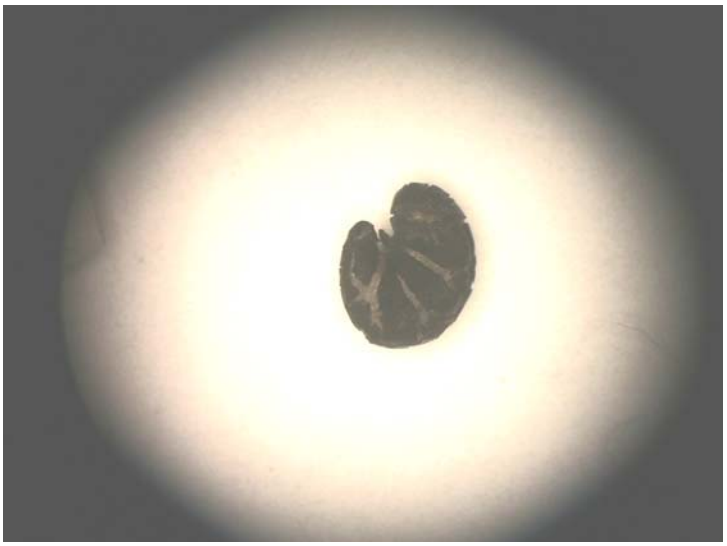


Image A.7: Control seed processed with SEM techniques observed under LM exhibits cracking.

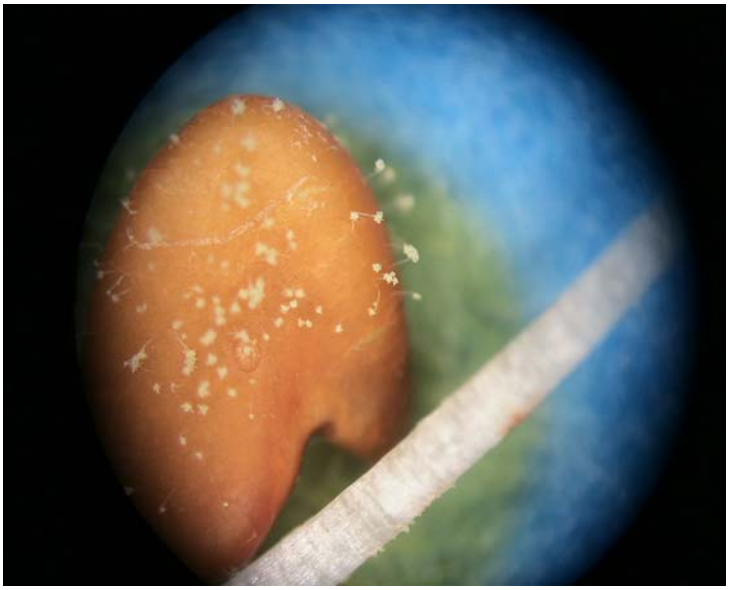
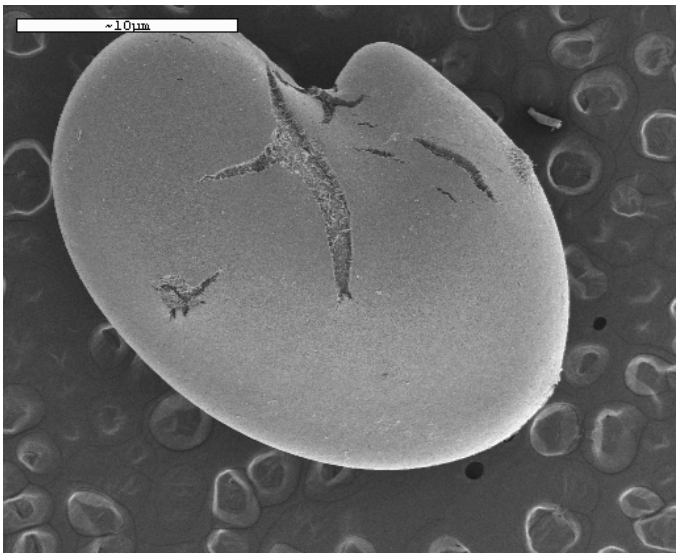


Image A.8: *Aspergillus* treated seed under LM exhibits minor cracking in the form of stress fractures due to cotyledon growth.



Asp D4: 12kV 30X Spot 6 WD 39 Ap3

Image A.9: *Aspergillus* treated seed under SEM exhibits minor indistinguishable cracking.