

Seed isolates of *Alternaria* and *Aspergillus* fungi increase germination of *Astragalus utahensis*

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ABSTRACT

Astragalus utahensis (Torr.) Torr. & A. Gray (Fabaceae) (Utah milkvetch) is native to the arid Great Basin and has desirable attributes that make it a good candidate for restoration in arid, noncompetitive situations. Seed dormancy is a significant barrier to consistent establishment for this species. Species of *Alternaria* and *Aspergillus* fungi have potential to enhance germination of *A. utahensis* seed; therefore, we conducted trials to investigate *Alternaria* and *Aspergillus* effects on germination and emergence under controlled *in vitro* conditions or in soil in a growth chamber, in a greenhouse, and in the field. Seed was either acid scarified or left untreated and then inoculated with spores from *Alternaria* and *Aspergillus*. Under *in vitro* and greenhouse conditions, rates of germination or emergence increased significantly for seed inoculated with the 2 fungi. Inoculated seed in field experiments planted at Fountain Green and Nephi, Utah, had significantly higher emergence rates than the non-scarified/non-inoculated control, and *Aspergillus*-inoculated seed outperformed seed treated with *Alternaria*. Inoculation of seed planted at Spanish Fork did not provide an advantage over acid scarification, but all treatments showed greater emergence than the non-scarified/non-inoculated control. This study demonstrates that inoculating *A. utahensis* seed with *Alternaria* or *Aspergillus* prior to planting has a positive impact on rates of emergence in a field setting.

Eldredge SD, Geary B, Jensen SL. 2016. Seed isolates of *Alternaria* and *Aspergillus* fungi increase germination of *Astragalus utahensis*. *Native Plants Journal* 17(2):89–94.

KEY WORDS

fungal–seed interactions, seed germination, Fabaceae

NOMENCLATURE

Plants: USDA NRCS (2015)

Fungi: Rotem (1994)

Photos by Brad Geary

Astragalus utahensis (Torr.) Torr. & A. Gray (Fabaceae) (Utah milkvetch) is an important habitat component of steep slopes and rocky areas of the Great Basin (Welsh 1986). This beneficial native plant often grows in disturbed areas with minimal competition but has difficulty competing against invasive plant species that tend to inhabit similar sites. Populations of Utah milkvetch provide early-season, non-toxic, non-bloating forage for animals; nitrogen fixation; and adaptation to the arid environments of the Great Basin. Therefore, use of *A. utahensis* in the reclamation of arid, disturbed lands is highly desirable. Effective establishment of Utah milkvetch necessitates the use of tools to enhance germination and emergence of seed that commonly exhibits strong dormancy characteristics.

Seed dormancy in the genus *Astragalus* is influenced by multiple factors, including temperature, moisture, water potential, seed source, presence of leachable inhibitory chemicals, and seed hardness (Baskin and Quarterman 1969; Ziemkiewicz and Cronin 1981). *Astragalus cicer* L. (chickpea milkvetch) seed germination is low because of the impermeability of the seedcoat (Miklas and others 1987). Baskin and Quarterman (1969) identified the outer and inner seedcoat of *A. tennesseensis* A. Gray ex Chapm. (Tennessee milkvetch) as impervious to water and constraining to the embryo. A chemical inhibitory substance was also recognized by Baskin and Quarterman (1969) to reduce germination, but it was a minor inhibitor compared to the seedcoat. Without physical or chemical triggering mechanisms, seed could remain dormant for several years (Baskin and Quarterman 1969). To use *A. utahensis* in restoration efforts, dormancy must be mitigated and conditions provided for seed to germinate quickly (Evenari 1962; Baskin and Quarterman 1969). Jensen (2004) observed that *A. utahensis* seed infected with a fungus germinated more rapidly than seed that was not infected. The fungi were identified as *Alternaria* and *Aspergillus*, and it was hypothesized that the presence of these or other fungi may stimulate *A. utahensis* germination and improve recruitment during restoration efforts.

Microbial activity on a seed can function as a triggering mechanism for germination through the breakdown of exogenous dormancy barriers, thereby accelerating normal germination patterns (Guttridge and others 1984; Rheeder and others 1990). Rheeder and others (1990) reported a positive correlation between corn germination and *Fusarium subglutinans* and other unidentified fungi. Guttridge and others (1984) reported significant increases in germination of strawberry seed inoculated with the fungus *Ulocladium charatarum* (74%) compared to the control (7%). Fungal interactions with *Astragalus* seed germination may aid in breaking dormancy and creating more uniform germination, thus giving inoculated seed a competitive advantage.

Alternaria and *Aspergillus* fungi have been shown to enhance germination in *A. utahensis* (Eldredge 2007). Each

genus, *Alternaria* and *Aspergillus*, belongs to the Deuteromycetes (imperfect) class of fungi and both have ubiquitous distribution (Rotem 1994; Agrios 2005). These genera adapt to a broad range of ecological niches and may act as either saprophytes or parasites (Spooner and Roberts 2005).

Alternaria is described as either parasitic or saprophytic, and many factors—including moisture, nutrient availability, and temperature—influence its growth and pathogenicity (Rotem 1994; Agarwal and Sinclair 1997). It is a weak parasite that will infect a host when the host is wounded and when a moisture-rich environment provides the optimal situation for infection (Rotem 1994). *Aspergillus* is considered a saprophyte despite its association with a variety of plant diseases, and its growth can be influenced by a number of environmental factors, such as moisture, temperature, and pH (Cotty and others 1994; Kozakiewicz and Smith 1994).

Initial studies suggest the possibility that *Alternaria* and *Aspergillus* fungi may effectively be used to manipulate seed dormancy in Utah milkvetch (Jensen 2004). To test this idea, a series of experiments were designed to 1) determine the influence of *Alternaria* and *Aspergillus* on inoculation on germination response in *A. utahensis* under carefully controlled *in vitro* culture conditions; and 2) examine effectiveness of *Alternaria* and *Aspergillus* on emergence of inoculated seed in a growth chamber, greenhouse, and field environments.

MATERIALS AND METHODS

Astragalus utahensis seed, collected at 1483 m (4865 ft) elevation near Spanish Fork, Utah, during June 2002 was subsequently cleaned and stored at 21 °C (70 °F). Seed used for trials was scarified in 98% sulfuric acid for 20 min to enhance imbibition and eventual germination, then immediately rinsed in double-distilled water for 10 s and maintained aseptically in a laminar flow hood until used in the trials.

Alternaria and *Aspergillus* cultures used in this study were isolated from indigenous *A. utahensis* seed collected from Spanish Fork Canyon, Utah County. Isolates were collected and maintained on potato dextrose agar (PDA) at 21 °C (70 °F). Light microscopy was used to observe the conidiophores and conidia and to confirm identification at the genus level.



Light microscopy of an *Astragalus utahensis* seed following acid scarification and inoculation with *Alternaria*. Seedcoat was cracked (arrows) and mycelium covered the seed.

Light microscopy of an *Astragalus utahensis* seed following acid scarification and inoculation with *Aspergillus*. Seedcoat was cracked (arrows) and conidiophores grew out of the seed and produced spores.



Acid-scarified and non-scarified seed were inoculated with either *Alternaria* or *Aspergillus* spore solutions created from 6-wk-old fungal cultures grown on a weak nutrient media of 1/3 strength PDA. Inoculum contained 10^6 spores/ml (33,814 spores per oz) of both fungi, as determined by hemacytometer counts. A drop of Tween 20 was added to each batch of inoculum. Scarified and non-scarified seed were flood-inoculated in polypropylene cone sieves by pouring the inoculum over the seed 3 times.

In vitro culture trials employed 20 randomly selected seeds per Petri plate for each of the 6 treatments: scarified, scarified *Alternaria*-inoculated, scarified *Aspergillus*-inoculated, non-scarified, non-scarified *Alternaria*-inoculated, and non-scarified *Aspergillus*-inoculated. Treatments were randomized within a block (grouping of each experimental treatment) and each block replicated 4 times (repeated over time). All seed was placed on 8.3 cm (3.3 in) diameter seed germination blotters (Anchor Paper, St Paul, Minnesota) and wetted with 7 ml (0.24 oz) of reverse-osmosis treated water. Petri plates were sealed with Parafilm and kept in a dark growth chamber at $15 \pm 2^\circ\text{C}$ ($59 \pm 3.6^\circ\text{F}$). Daily cumulative germination counts were recorded through day 11, with germination defined as visible extension of the radicle.

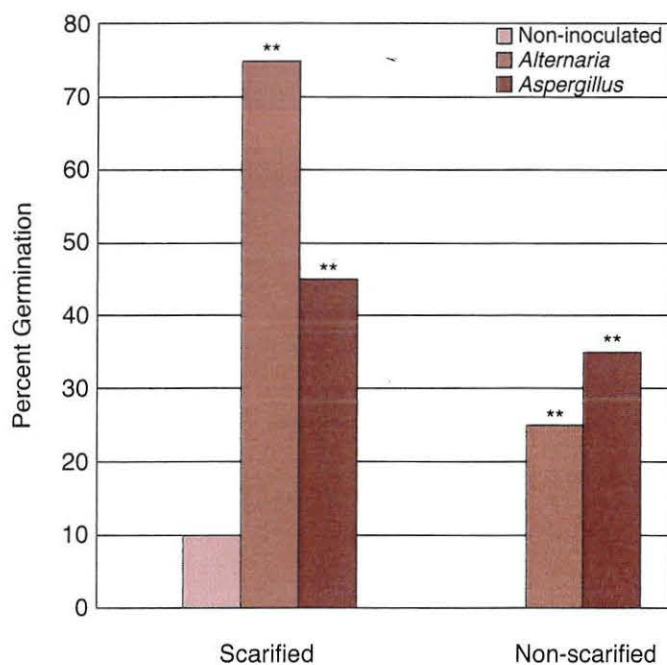
Growth trays of 66 cells (6×11 cells, Spencer_Lamier #90-6) were used for growth chamber and greenhouse emergence trials. Each tray was sterilized with 10% sodium hypochlorite and filled with Sunshine #1 potting soil (SunGro Horticulture Canada, Seba Beach, Alberta). Groups of 18 randomly selected, acid-scarified seed from the control, the *Alternaria*-inoculated, and the *Aspergillus*-inoculated treatments were planted one seed per cell with all treatments within a tray. Seed was planted 0.65 cm (0.25 in) deep. A single row of unplanted cells was left between each treatment to prevent cross contamination. The growth chamber trial was designed with 4 blocks per replication, and the study was repeated twice. The greenhouse trial had 5 blocks per replication and was repeated 3 times.

Planted trays in the growth chamber were kept inside a clear plastic covering to maintain relative humidity around 90%. After

1 wk, the clear plastic covering was removed. Growing conditions were 21°C (70°F), with a 12-h light/dark cycle, and bottom watering. Cumulative emergence counts were recorded daily for 30 d.

Planted trays in the greenhouse study were misted for 8 sec every 6 min for 7 d and were then moved to a non-misted bench. Growing conditions were approximately 26.6°C (80°F) during the day and 15.5°C (60°F) at night. Subsequent watering occurred every 3 to 4 d, or as needed, with no supplemental nutrients. Germination counts were recorded daily for 9 d, and cumulative germination was recorded 30 d after planting.

Field trials were planted at 3 sites: near Fountain Green, Nephi, and Spanish Fork, Utah. The Fountain Green field site is located east of the San Pitch Range at an altitude of approximately 1800 m (5905 ft). The Nephi site is located on an open valley floor at an altitude of approximately 1500 m (4921 ft). The Spanish Fork site is located on the west side of the Wasatch Range at an altitude of approximately 1300 m (4265 ft). Planting occurred on 2 different dates, 6 mo apart—25 September 2006 and 25 March 2007. The 4 treatments included *Aspergillus*-inoculated; *Alternaria*-inoculated; acid-scarified, non-inoculated; and no acid scarification, non-inoculated (control) seed. We used 30 seeds per treatment per block, and each site incorporated 5 blocks. *Aspergillus*- and *Alternaria*-inoculated seed was dried for 12 h following inoculation, then had 3 wk of storage at 5°C (41°F). A Hege 1000 series drill (Hege, Colwich, Kansas) planted all seed. The drill was sterilized with 98%



** P value < 0.0001 when compared to the non-inoculated seed.

Figure 1. Percent germination of *Astragalus utahensis* seed under controlled, *in vitro* conditions. Seed was treated combinatorially by either acid scarification with sulfuric acid or no scarification and either inoculation with *Alternaria* or *Aspergillus* spores or no inoculation.

ethanol between treatments to minimize cross-contamination. We made emergence counts after 6 mo for the fall-planted plots and after 3 mo for spring-planted plots.

In vitro, growth chamber, greenhouse, and field trials incorporated a randomized complete block design. Data from all trials were analyzed using Poisson regression using NCSS software (NCSS Statistical Software, Kaysville, Utah; 2001). An alpha level of 0.05 was set to define statistical significance.

RESULTS AND DISCUSSION

Scarified seed treated with *Alternaria* or *Aspergillus* under *in vitro* controlled conditions showed a significant increase in germination over those treated with water (Figures 1, 2A,B,C). Acid-scarified, non-inoculated seed germinated at a rate of 10%, while *Alternaria*- and *Aspergillus*-inoculated treated seed germinated to 75% and 45%, respectively. These fungi also sig-

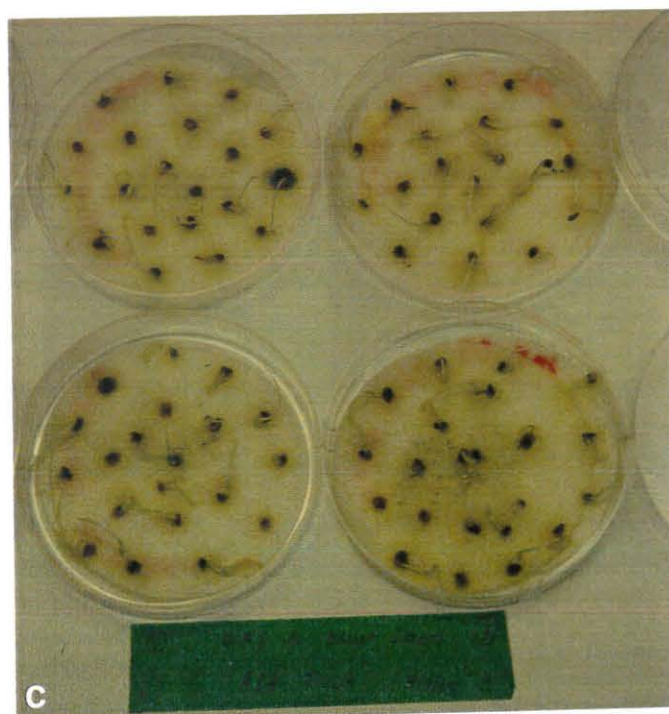
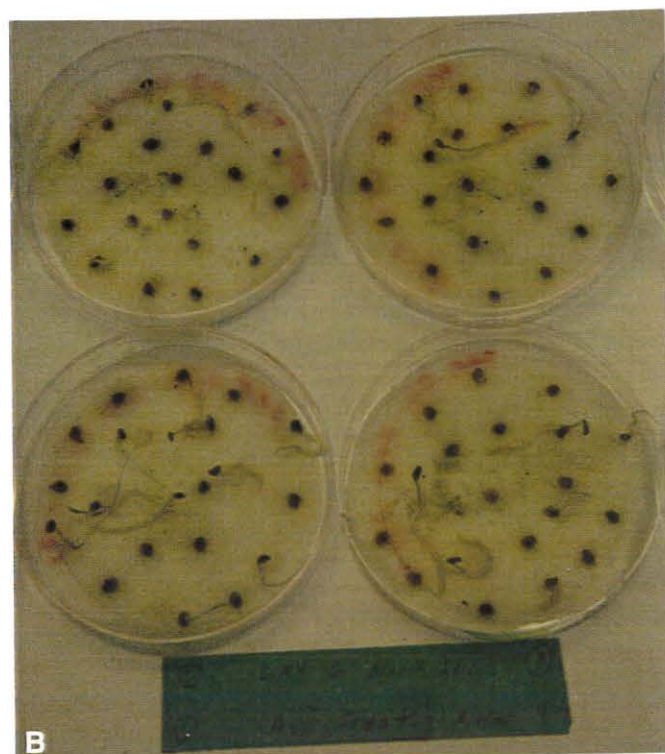
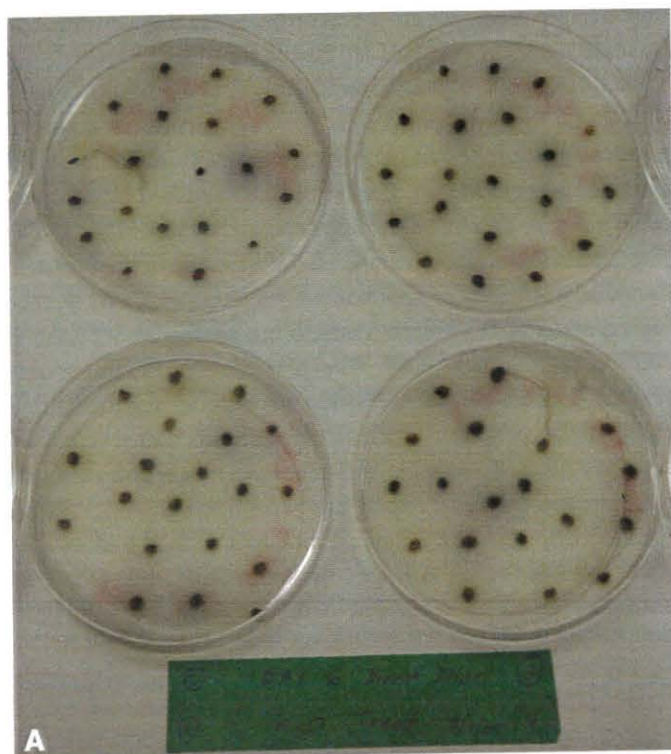


Figure 2. *Astragalus utahensis* seed 6 d into germination evaluations: non-scarified seed germinated without fungal inoculant (A); non-scarified seed inoculated with *Aspergillus* spores (B); and non-scarified seed inoculated with *Alternaria* spores (C).

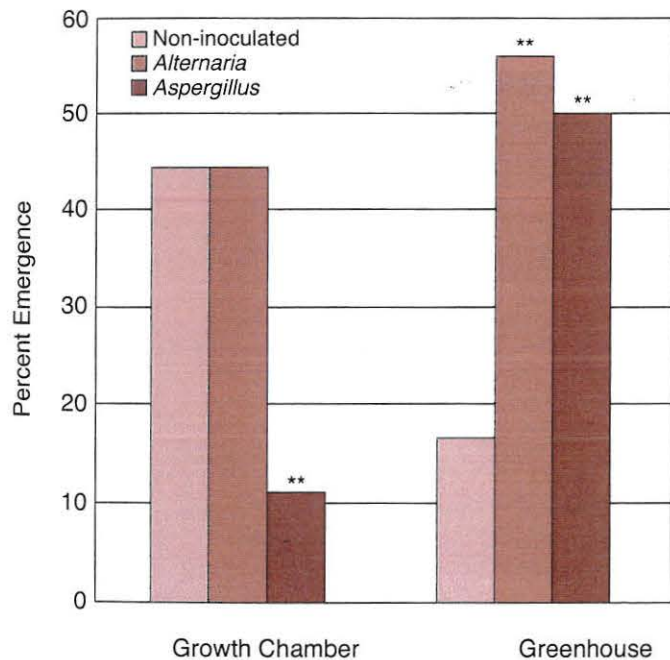
nificantly increased germination when the seed was not acid scarified. The non-inoculated non-scarified seed did not germinate. In comparison, 35% and 25%, respectively, of non-scarified *Aspergillus*- and *Alternaria*-inoculated seed germinated (Figure 1). Acid scarification clearly increased germination, probably by weakening seedcoat barriers characteristic of *A. utahensis*, but the fungi also increased germination through an unidentified mechanism.

Results from the growth chamber trial were distinct from the other *in vitro* germination and emergence studies. No statistical differences existed between the *Alternaria*-inoculated and non-inoculated treatments; both treatments germinated to 44% (Figure 3). *Aspergillus*-inoculated seed emerged at a rate of 11%, significantly lower than the rate for the non-inoculated treatment. The reasons for this unique response are unknown but may have been related to the enclosed, humid chamber environment that kept soil and atmospheric moisture high. The increased moisture levels may have increased emergence of seed or increased seedling disease, which changed the seed response.

Greenhouse trial results mirrored the *in vitro* study results in that non-inoculated acid-scarified seed emerged at a rate of 17% while the *Alternaria*- and *Aspergillus*-inoculated seed was significantly higher at 55 and 50%, respectively (Figure 3). *Alternaria*- and *Aspergillus*-inoculated seed also differed significantly from each other ($P = 0.0106$) in rate of emergence. The

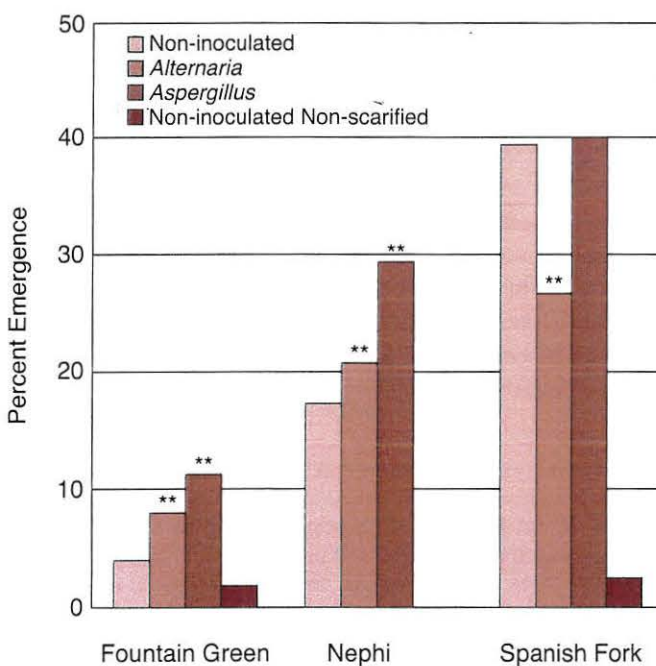
added environmental variables (namely soil) associated with the greenhouse trials as opposed to the *in vitro* trials demonstrated the ability of *Alternaria* and *Aspergillus* to increase emergence of *A. utahensis* seed in either controlled or variable environments.

The spring-planted (2007) field trials at Fountain Green, Nephi, and Spanish Fork failed to provide usable results. No seed emerged for any treatment, most likely the result of low soil moisture conditions. Seed planted during fall 2006 successfully emerged, and the Fountain Green and Nephi site results were similar to those seen with the *in vitro* and greenhouse trials. *Alternaria*- and *Aspergillus*-inoculated seed produced significantly higher emergence rates than both the control acid-scarified and non-scarified seed. At Fountain Green, *Alternaria*-inoculated seed emerged at a rate of 8% and *Aspergillus*-inoculated at 11% as compared with 4% and 1.5% for acid scarified and non-scarified seed, respectively. At Nephi, *Alternaria*-inoculated seed emerged at a rate of 21% and *Aspergillus*-inoculated at 29% as compared with 17% and 0% for acid scarified and non-scarified seed, respectively (Figure 4). *Aspergillus*-inoculated seed outperformed *Alternaria*-inoculated seed in the field, even though the reverse was true in the *in vitro* and greenhouse experiments. This result is not necessarily surprising considering that *Alternaria* proliferates in areas with mild temperatures and high moisture, conditions common to those imposed by the *in vitro* and greenhouse



** P value < 0.0001 when compared to the non-inoculated seed.

Figure 3. Percent emergence of scarified *Astragalus utahensis* seed planted in soil under growth chamber and greenhouse conditions. Seed was scarified with sulfuric acid and either not inoculated or inoculated with *Alternaria* or *Aspergillus* spores.



** P value < 0.0001 when compared to the non-inoculated seed.

Figure 4. Percent emergence of *Astragalus utahensis* seed planted at field locations near Fountain Green, Nephi, and Spanish Fork in fall 2006. Seed was scarified with sulfuric acid and either not inoculated or inoculated with *Alternaria* or *Aspergillus* spores, or seed was both not scarified and not inoculated.

environments (Rotem 1994). *Aspergillus* may have performed poorly in these environments because, although it has adapted to a wide range of temperatures, it performs best in arid conditions (Cotty and others 1994).

Aspergillus-inoculated seed again produced higher emergence rates (40%) than did *Alternaria*-inoculated seed (28%) at the Spanish Fork site; however, emergence for *Aspergillus*-inoculated seed was not significantly different from acid-scarified seed (39%) (Figure 4). *Alternaria*-inoculated seed had significantly less emergence than did acid-scarified seed, and non-scarified seed had the lowest germination at 3.4% (Figure 4). Overall, at Spanish Fork all treatments produced higher emergence than did corresponding treatments at the other locations. The nature of the study did not elucidate the reasons for this discrepancy, but one possible reason is elevated moisture conditions that may have obscured the fungi effects at this location, similar to what was seen in the growth chamber study.

Scarified seed treated with *Alternaria* or *Aspergillus* under *in vitro*, greenhouse, and fall-seeded plots near Fountain Green and Nephi had significant increases in germination of Utah milkvetch seed, confirming observations that *A. utahensis* seed infected with a fungus germinated more rapidly than seed that was not infected. Inoculated seed may provide competitive assistance in the reclamation of disturbed lands in the arid environments of the Great Basin.

Effective establishment of Utah milkvetch would provide forage for animals and would help minimize the deleterious effects of invasive species. Germination results from *Alternaria* or *Aspergillus* inoculation may be further improved if seed was primed or coated with a polymer that retains moisture; however, further research is needed.

ACKNOWLEDGMENTS

Funding for this study was provided by the USDA Forest Service Great Basin Native Plant Selection and Increase Project, the USDA BLM Great Basin Restoration Initiative, and Brigham Young University.

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