

THE EFFECTS OF SUPPRESSIVE BACTERIA ON THE GERMINATION AND
GROWTH OF CHEATGRASS (*Bromus tectorum* L.)

By

AMANDA KAY HOHNHORST

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of AMANDA KAY HOHNHORST find it satisfactory and recommend that it be accepted.

Rodney D. Sayler, Ph.D., Chair

Linda H. Hardesty, Ph.D.

Lisa A. Shipley, Ph.D.

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Abstract

by Amanda Kay Hohnhorst, M.S.
Washington State University
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Chair: Rodney D. Sayler

Cheatgrass is a problematic invasive annual species that is a major factor in the deterioration of our native shrub steppe communities and has substantially increased the frequency of costly wildfires across the western states. We tested the effects of the bacterium, *Pseudomonas fluorescens* strain D7, on the germination and long-term growth of cheatgrass (*Bromus tectorum* L.) seedlings in a greenhouse experiment conducted at Washington State University. The D7 inoculum treatment reduced early germination of seeds over approximately the first 7 days of growth, but long-term germination across 35 days was not significantly reduced. Germination varied significantly by seed accession as well as by interaction effects with time and inoculum treatment, indicating a potentially complicated series of ecological and environmental interactions. Cheatgrass seedlings grown in pots treated with D7 had lower total root weights, but higher shoot weights per pot, which may represent compensatory and adaptive growth responses to the stress of the D7 bacterium. However, variation in different seedling characteristics (i.e., root weight, total root and shoot weights, and longest root measurements) among seed accessions was significant and differences among accessions had the largest explanatory power in

statistical models and the greatest influence on germination and seedling characteristics. These presumed ecological variations by geographic location may represent differences in adaptations to local environmental conditions. The resulting ecological variations in response to D7 should be considered in future greenhouse and field trials to more precisely determine the potential for using D7 to control cheatgrass in natural settings over a wide geographic expanse.

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Dedication

This thesis is dedicated to my daughter.

THE EFFECTS OF SUPPRESSIVE BACTERIA ON THE GERMINATION AND GROWTH OF CHEATGRASS (*Bromus tectorum* L.)

INTRODUCTION

The introduction and spread of invasive species is an ever-increasing challenge as we strive to ensure the preservation of native species and biological diversity. Cheatgrass (*Bromus tectorum* L.) or downy brome is an invasive winter annual grass native to southern Europe and southwestern Asia. Cheatgrass was first introduced into North America before 1790 with shipments of grain or interspersed in filling materials used to protect packed goods (Mack 1981; Novak 2004). Distribution expanded within the western United States by the 1890's and was further hastened via livestock, contaminated grain, and railroads (Mack 1981; Billings 1994). By 1946, cheatgrass had already occupied an estimated 4 million ha in eastern Oregon and over 5 million ha by 1990 in Idaho and Utah (Monsen 1994; Pimentel et al. 2005). More recently, Duncan et al. (2004) estimated a 14% annual spread rate with over 22 million ha infested in 17 western states. In the future, climate change and increased CO₂ production could play a major role in advancing the spread of this alien species even further, and predictive models have been developed to aid in addressing this issue (Thuiller et al. 2007; Bradley 2009).

The invasion and subsequent development of dense, monoculture stands of cheatgrass found throughout the western rangelands create an unprecedented ecological impact by directly increasing wildfires, soil erosion, and the deterioration of native shrub steppe communities (Whisenant 1990, Beyers 2004, Mack 1981).

Indirectly, this ecological damage continues to have a variety of environmental impacts such as the loss of wildlife habitat and populations and decreased water quality, productivity in crop and rangelands, and property value (Pellant 1990; Knapp 1996). Every year government agencies spend millions of dollars battling cheatgrass-fueled wildfires in addition to the post-fire rehabilitation or pre-suppression costs. In 1996, Knapp estimated annual fire cost attributed to cheatgrass to be \$10 million for the Great Basin region. It has also been estimated that wheat farmers alone bear approximately \$335-370 million in lost yields and herbicide expenses when dealing with this invasive species (Gurusiddaiah et al. 1994). There is little doubt that the current cost of the cheatgrass invasion is even higher than these approximations given the time frame of the above estimates, in addition to poorly quantified indirect costs. Cheatgrass does, however, have some benefit as an early season forage for livestock and wildlife although there is great variability in annual production, and once barbed awns are present, it becomes unpalatable by causing sore mouths (Morrow and Stahlman 1984; Knapp 1996; Reid et al. 2008). Therefore the costs of cheatgrass far exceed the forage benefits and continued efforts should be made towards suppressing and eradicating this species where feasible.

Cheatgrass Ecology

Cheatgrass has many characteristics that contribute to its spread and persistence on the landscape. Upon arrival, it was pre-adapted to the typical hot, dry summers and cold winters of the intermountain West (Mack 1981; Knapp 1996). Cheatgrass also has the ability to germinate at low temperatures in the fall and spring, thereby increasing its chances of survival while simultaneously maintaining a

competitive edge (Mack and Pyke 1983; Monsen 1994). Fall germination and rapid, dense root elongation guarantee access to soil nutrients and moisture throughout the winter before native perennials develop (Morrow and Stahlman 1984; Thill et al. 1984; Monsen 1994). If germination occurs in the fall, plants mature much earlier in the summer, dry out quickly, and produce fine-fuel continuity across the terrain that has proven to be a major fire hazard (Whisenant 1990). During this time, copious amounts of barbed awns that easily attach to fur and clothing are dispersed for maximizing reproductive success (Morrow and Stahlman 1984). If conditions in spring are more favorable for germination, cheatgrass remains highly competitive in sequestering resources that would otherwise be used by indigenous species. Sites disturbed from fire, overgrazing, and other human activities provide the perfect open opportunity for colonization. Cheatgrass seeds have the ability to survive fires and take full advantage of these cleared sites for initial occupation or recolonization. Consequently, fire regimes in the western regions have been altered greatly by increased fuel connectivity, longer fire seasons, decreased species diversity, and more frequent fires (Whisenant 1990). Whisenant (1990) determined that fire frequency has dramatically increased from 60-110 year intervals historically, to less than every 5 years post-invasion, thus aiding in further proliferation. All of the above characteristics enable cheatgrass to be a persistent competitor on the landscape and virtually impossible to eradicate once well established across large areas.

Historically, cheatgrass eradication efforts have been well-studied and extensively employed in field settings. Several methods for controlling invasive weeds including burning, grazing, chemical applications, mechanical removal, alteration of

cultural practices, and biological control agents have been developed and used for controlling brome grasses (Pellant 1996; DiTomaso 2000). Currently, biocontrol fungal pathogens such as *Ustilago bullata* (head smut), *Tilletia fusca* (chestnut bunt), and *Pyrenophora semeniperda* (black fingers of death) are of particular interest and have demonstrated positive results in either preventing seed production or killing mature cheatgrass seeds that have already developed (Meyer et al. 2008). Likewise, the rhizobacteria, *Pseudomonas fluorescens* strain D7, is another potential biological control agent that has demonstrated effective results in producing phytotoxins and suppressing growth of downy brome (Kennedy et al. 1991). Although the exact mechanism is not entirely known, the destabilization of membranes and lipid production have been considered as the main inhibitory factors (Tranel et al. 1993; Gurusiddaiah et al. 1994). Few studies have examined the effect of directly applying D7 to the soil rhizosphere and observing the growth of cheatgrass for any extended period of time. We evaluated biotype variability (i.e., seed accession) and the long-term effectiveness of D7 application in suppressing cheatgrass germination and root and shoot growth in a controlled greenhouse setting.

MATERIALS AND METHODS

Seed Collection

Cheatgrass seed was collected from six different ecological sites at varying elevations from July through September in 2006. All six sites were of the sagebrush-grassland vegetation type with prominent cheatgrass found in the understory. These sites were evenly classified into two geographical subgroups, northern and southern.

The northern group consisted of three areas located near where D7 was first isolated (Kennedy et al. 1991), including sites near Pendleton, OR (P), Lind, WA (LI), and La Crosse, WA (LA). For the southern group, seed was collected at Dubois, ID (D), Ada County, ID (A), and Harney County, OR (H). Seeds were collected from the soil surface directly beneath mature cheatgrass plants covering about 0.4 ha at each of the collection sites. Collected seed was placed in paper bags to absorb any moisture and transported in coolers at ambient temperature.

Planting and Inoculation

Before planting, seed germination trials were conducted to test viability. With a 100% germination rate, four seeds were selected for planting in each container. Cheatgrass seeds were then planted at soil surface in standard 3.8 l greenhouse pots containing pre-moistened, local sieved Palouse silt loam soil. The inoculum D7 was obtained from the United States Department of Agriculture, Agricultural Research Center, at Washington State University where it was grown in a fermentation solution. It was applied directly to the soil surface via pipette around the cheatgrass seeds immediately after planting. A 3 mm layer of sterilized sand was evenly dispersed over the seeds to prevent shifting during watering.

Greenhouse Conditions and Harvest

Seedlings were watered as needed to maintain damp soil at field capacity using tap water 2 - 3 times a week without fertilizer additives. Pots were rearranged at each watering to randomize their location within the greenhouse to minimize differences in growing conditions. Greenhouse temperatures were maintained on a 20/20°C (day/night) regime. Germination date was recorded for each seedling and mature

plants were harvested at peak standing crop after 101 days of growth. Roots and shoots were washed with tap water and separated. Root length was recorded and all samples were later dried in an oven for 48 h at 20°C to obtain dry weight data.

Statistical Analysis

Statistical analyses were conducted using JMP 7.0 (SAS Institute, Inc. 2007). We used a split plot repeated measures design to evaluate the effect of the experimental treatment (inoculum vs. no inoculum) on seed germination as well as the ancillary categorical variable of accession (six seed collection locations). Ten replicated pots for each treatment were planted with four cheatgrass seeds. In the repeated measures design, germination (total seeds germinated, 0 - 4) was measured 10 times for each pot at 2 – 7 day intervals over the first 35 days of plant growth. The Fit Model platform in JMP uses a multiple analysis of variance (Manova) fitting personality that fits mixed models using modern restricted maximum likelihood (REML) computational methods and Kenward-Rogers tests (SAS Institute, Inc. 2007). For other response variables, we used a least squares (Anova) mixed model (SAS Institute, Inc. 2007) with the inoculum treatment and accession as fixed factors and individual planting pot as a random factor when appropriate (e.g., root and shoot weights per plant). Length of the longest root was recorded as a single measurement for each pot at the end of the experiment. Weight of roots, shoots, and the total weight of roots and shoots combined were measured per seedling per pot at the end of the growing experiment. Lengths were measured to the nearest mm and weights were recorded as ± 0.01 g. The frequency distributions of variables approximated normality, and consequently, the

modeling results using either raw or log-transformed variables were equivalent. Therefore, we report the results obtained from using untransformed variables.

RESULTS

Seed Germination

Germination of cheatgrass seeds over 35 days of repeated measurements varied significantly between subjects (i.e., across pots) by accession but not inoculum treatment (Table 1). The repeated measures for time effects within subjects (pots) were significant for the variable time as well as all interactions between time, accession, and inoculum, indicating a complicated series of interactions, which were subsequently explored further in analyses for individual time periods. Overall, the D7 bacterium treatment reduced cheatgrass seed germination by about 28% during the first 7 days of the germination period, after which germination rates converged over time (Fig. 1). Germination of seeds also varied across time by accession (Fig. 2). When the six seed collection sites were grouped by broad geographic location (north vs. south), a pattern emerged of seeds collected from southern locations germinating at higher rates than seeds from northern locations ($F = 7.01$, $DF = 1/117$, $P = 0.0092$) (Fig. 3).

Seedling Characteristics

The longest root per pot (inoculated: mean = 30.9, SE = 0.50; untreated: mean = 28.9, SE = 0.48) varied significantly by inoculum treatment ($F = 8.18$, $DF = 1$, $P = 0.0051$) and accession ($F = 3.36$, $DF = 5$, $P = 0.0074$), however, a geographic grouping of accessions into north and south groups was not significant ($P = 0.74$). Root weights for individual plants varied significantly by accession ($F = 4.16$, $DF = 5$, $P = 0.0109$), but

not by inoculum treatment ($P = 0.28$). The north vs. south geographic grouping of accessions also was not significant ($P = 0.32$) for root weights. Variation in root weights attributable to individual pots contributed about 10.5 % of the residual variance in the model. Shoot weights were greater ($F = 4.53$, $DF = 1/96.2$, $P = 0.036$) for inoculated ($x = 5.08$, $SE = 0.20$) vs. untreated seedlings ($x = 4.48$, $SE = 0.20$), but did not vary by accession ($P = 0.84$). Variation in shoot weights attributable to individual pots contributed about 4.1% of the residual variance in this model.

At the end of the growth experiment at 101 days, the total weight of roots per pot ($R^2 = 0.29$, $F = 3.99$, $DF = 11$, $P < 0.0001$) was not significantly related to inoculum treatment ($P = 0.29$), but varied significantly by accession ($F = 6.0$, $DF = 5/5$, $P < 0.0001$) and an accession x inoculum interaction ($F = 2.55$, $DF = 5/5$, $P = 0.032$). We explored this interaction in greater detail using an independent, nonparametric regression tree model, which produced a simple model that split total weight of roots per pot into three different accession groupings and one inoculum x accession interaction (Fig. 4). The model revealed the interaction effect as that of accession D having a greater total weight of roots per pot than other accessions, and within accession D, inoculated pots having lower total root weights than untreated pots (Fig. 4). In this model, accession accounted for about 72% of the explanatory power of the model and inoculum treatment about 28%.

The total weight of shoots per pot ($R^2 = 0.17$, $F = 3.68$, $DF = 6$, $P = 0.002$) varied significantly by accession ($P < 0.004$), but only marginally by inoculum treatment ($P < 0.08$). Similarly, an independent regression tree model revealed that total weight of shoots varied among three different accession groupings, and that within a grouping of

three accessions (D, H, P), inoculated pots had a higher total weight of shoots than other accessions (Fig. 5). In this model, accession accounted for about 86% of the explanatory power of the model and inoculum treatment about 14%. A measurement of total biomass, the total weight of roots and shoots combined per pot, did not vary either by accession ($P = 0.70$) or by D7 inoculum treatment ($P = 0.26$). In summary, the bacterium D7 had mixed effects on seedling germination and growth, or interactions with different seed accessions, and seed accession had a large influence on specific response to D7 (Table 2).

DISCUSSION

Seed Germination

We demonstrated that the D7 inoculum reduced early germination of cheatgrass by about 28% during the first week of germination, after which germination rates then converged during the 35-day experiment. Johnson et al. (1993) found that D7 readily multiplied in cool conditions, but had little effect in suppressing root growth at higher temperatures (25/15°C). Consequently, it is possible that the relatively constant greenhouse temperatures in our experiment (20/20°C) may have reduced the effectiveness of D7 in suppressing germination and contributed to the observed convergence of treated and untreated seedling germination rates over time. Testing the effects of D7 at different temperatures in future studies of cheatgrass germination and growth in greenhouses may improve understanding of the specific conditions that influence D7 performance in different environments.

The significant difference in germination rates among north vs. south seed accession groupings, and other observed differences in seedling characteristics (e.g., longest root, total root weights by accession, shoot weights per plant, total shoot weights per pot), may be due to environmental differences among the geographic locations sampled in this study. Cheatgrass is known to have high phenotypic plasticity and local populations would be expected to rapidly evolve local adaptations (e.g., timing of germination; adaptations to soils, moisture, and temperature regimes) that improve seedling survival and reproductive success (Kao et al. 2008). For example, Kao et al. (2008) found a faster germination rate in cheatgrass seeds from higher elevations. The influence of these types of local adaptations in influencing germination rates and response to D7 could be evaluated in greater detail in future greenhouse seed growth trials using soil from the collection sites and by replicating environmental conditions (e.g., soil temperatures) that reflect major geographic differences.

Seedling Characteristics

Cheatgrass seedlings grown in pots treated with D7 had lower total root weights, but higher shoot weights per pot. Although these responses occurred across different accessions and were measured on a per pot basis, they also may be linked responses within individual plants and could represent compensatory responses to physiological stress caused by D7. Plants may compensate for suppressed root growth by devoting more growth resources to producing shoots and photosynthetic surface area needed for energy production.

Although we found that the longest root per pot and root weights varied by accession, the comparison by a north vs. south accession grouping was not significant.

Consequently, unmeasured ecological variation potentially related to local environmental adaptations of different cheatgrass populations may be more important in explaining the observed differences among accessions in our greenhouse trials. It is possible that the potential effect of north vs. south geographic variation in root or shoot growth could have been lost due to the extensive growing season in the greenhouse. Differences in roots and shoots may have been greater if they could have been measured at earlier stages during the germination and growth process when the immediate influence of D7 appears to have been greater. There is convergence in germination rates over time, and so differences in seedling growth by treated and untreated may be greater earlier in the growth phase as well. Future investigations of the effect of D7 on growth of cheatgrass could take seedling measurements at predetermined intervals throughout the growth period to more fully describe time effects.

We conclude that D7 bacterium had a variety of influences on growth and development of cheatgrass seedlings in a greenhouse environment, and acted to suppress total root weights in some, but not all accessions, and increased shoot weights. However, variation in germination and growth responses among accessions was significant and differences among accessions had the largest explanatory power in statistical models and the largest influence on germination and seedling characteristics. Thus, these presumed ecological variations in response to D7 among seed accessions are important to consider and need to be studied in additional greenhouse and field trials to more precisely determine the potential for D7 to be used to control cheatgrass in natural settings.

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Table 1. Repeated measures analysis for germination of cheatgrass seeds over 35 days in a greenhouse when treated with the bacterium, *Pseudomonas florescence*, strain D7, Washington State University, 2007.

Between Subjects (across pots)	F Value	Numerator DF	Denominator DF	P value
All Between	5.97	6	112	0.0001
Intercept	1567.3101	1	112	0.0001
Inoculum	3.06	1	112	0.0830
Accession	6.57	5	112	0.0001
Within Subjects (time effects)				
Time	248.38	9	104	0.0001
Time x Accession ¹	2.40	45	468.32	0.0001
Time x Inoculum	1.76	9	104	0.0843

¹ Test = Wilk's Lambda.

Table 2. Summary of the statistical effects of the D7 bacterium, *Pseudomonas fluorescence*, on cheatgrass seedling germination and growth in a greenhouse, Washington State University, 2007.¹

<u>Variable</u>	<u>Effects of D7</u>	<u>Effect of Accession</u>
Germination:	Delayed Early Germination	Varied among accessions
Roots:		
- Longest Root	Increased Length	Varied among accessions
- Root Weights	N.S.	Varied among accessions
Total Root Weights	N.S.	Inoculum x Accession interaction (<i>accessions responded differently</i>)
Shoots:		
- Shoot Weights	Increased	N.S.
Total Shoot Weights	Increased (marginally)	Varied among accessions

¹ N.S. = not significant at $P < 0.05$

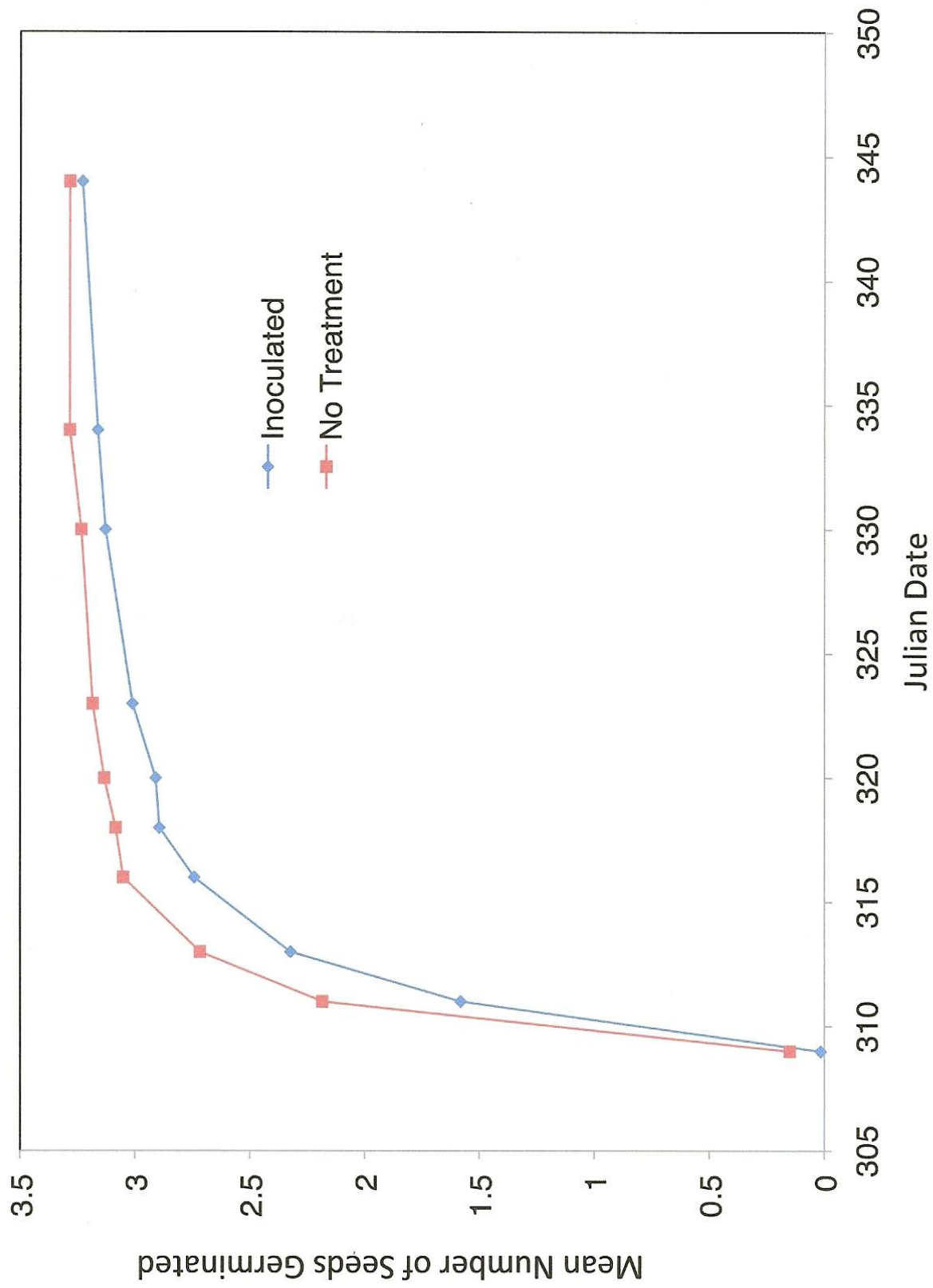


Fig. 1. Germination of cheatgrass seeds in a greenhouse when treated with the D7 strain of the bacterium, *Pseudomonas fluorescens*, Washington State University, 2007.

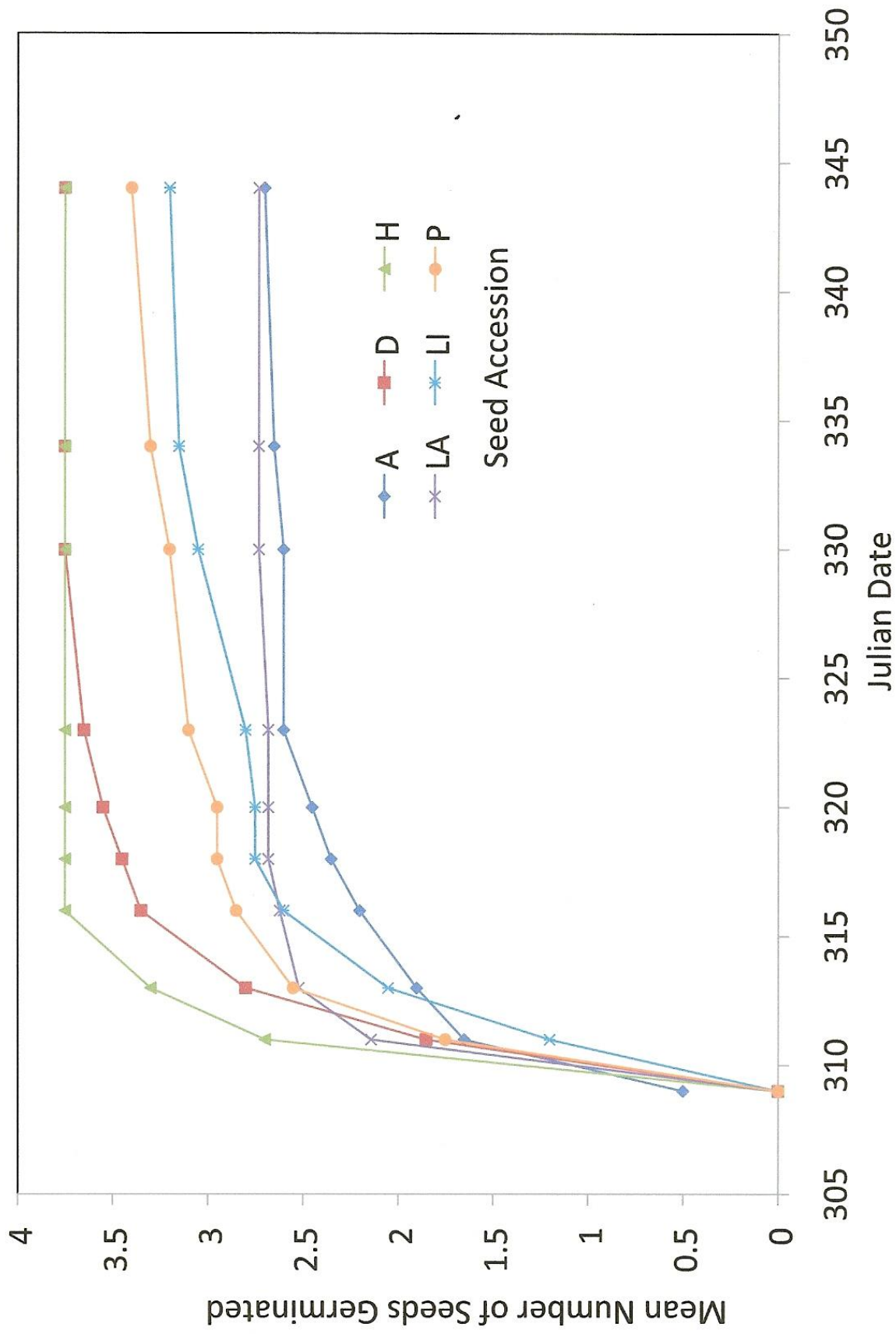


Fig. 2. Germination of cheatgrass from six different seed sources when grown in a greenhouse and treated with the D7 strain of the bacterium, *Pseudomonas fluorescens*, Washington State University, 2007.

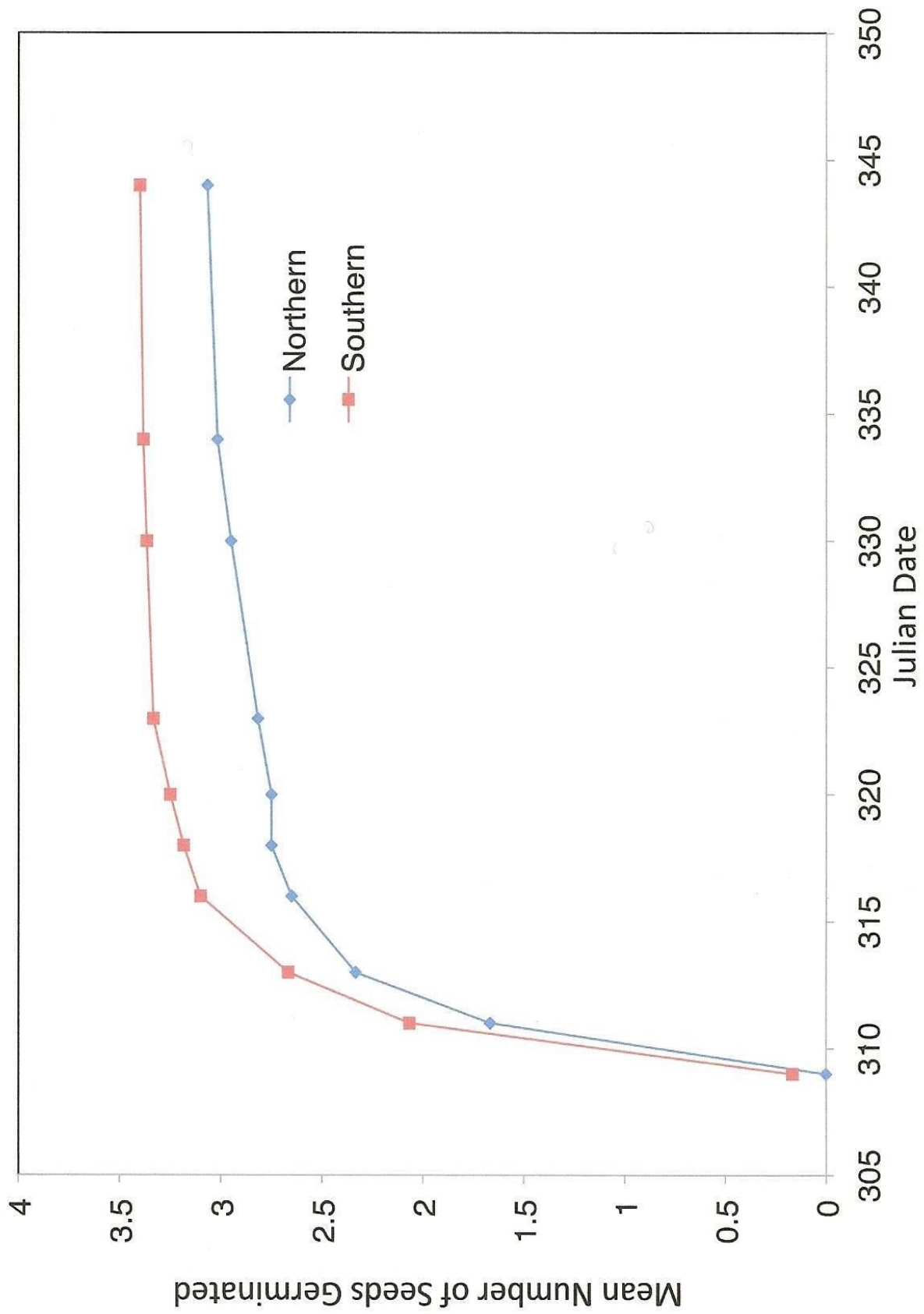


Fig. 3. Germination of cheatgrass seeds from northern and southern seed accessions when grown in a greenhouse and treated with the D7 strain of the bacterium, *Pseudomonas fluorescens*, Washington State University, 2007.

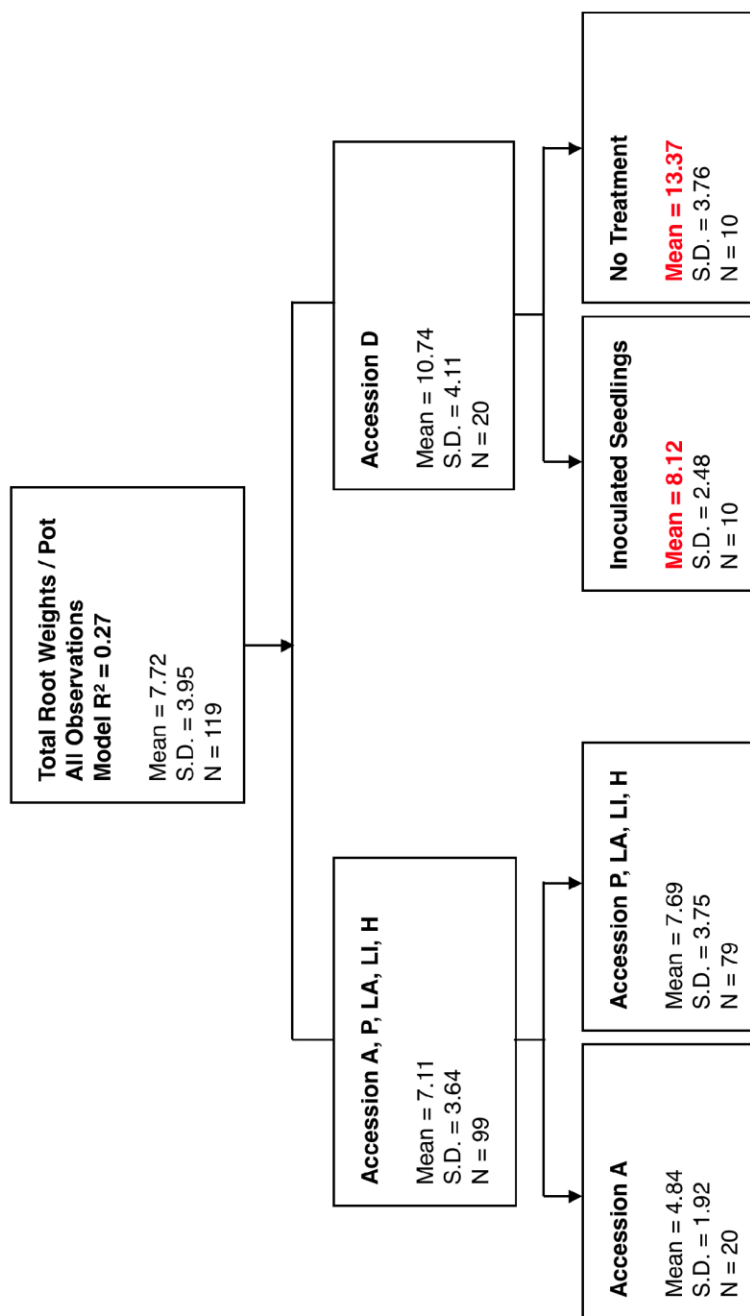


Fig. 4. Regression tree model of total weight (g) of cheatgrass seedling roots per pot when grown in a greenhouse and treated with the D7 bacterium, *Pseudomonas fluorescens*, Washington State University, 2007.

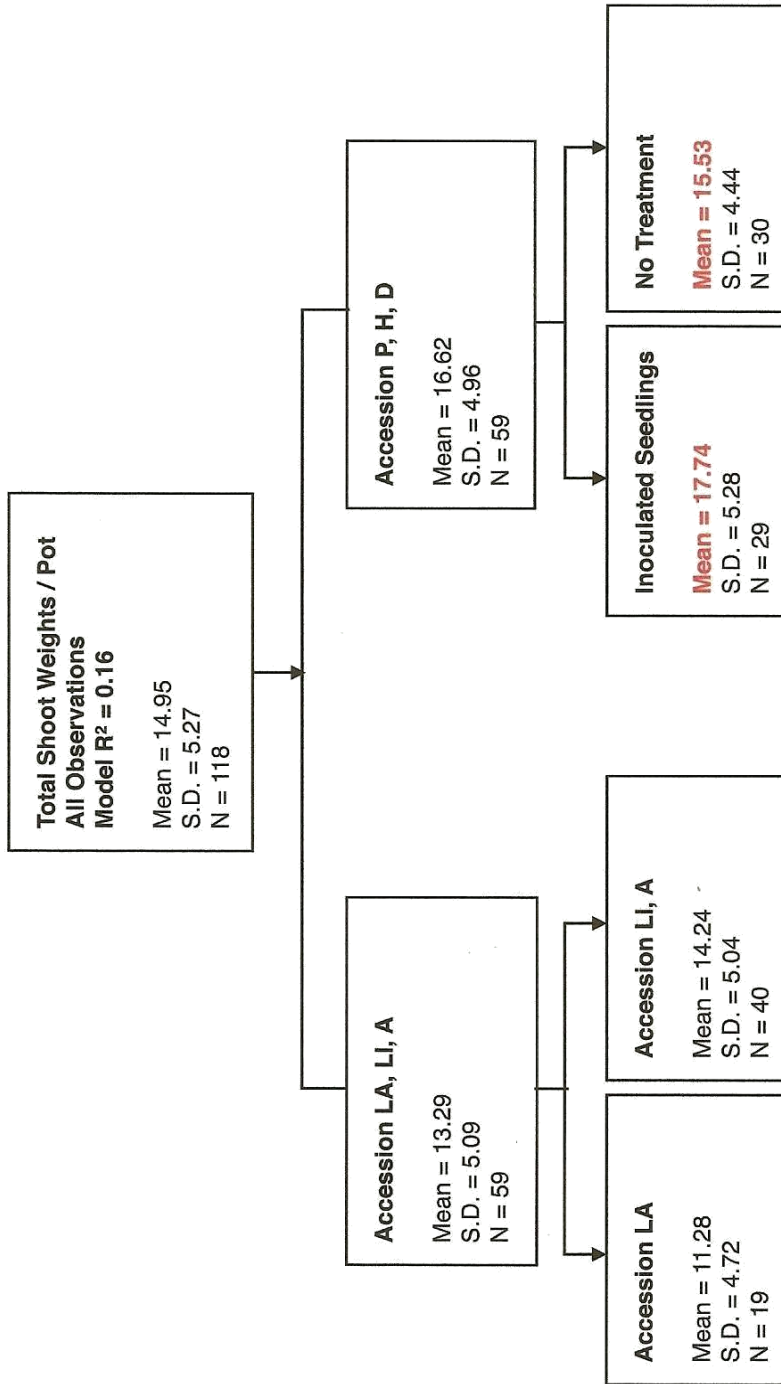


Fig. 5. Regression tree model of total shoot weights (g) of cheatgrass seedlings per pot when grown in a greenhouse and treated with the D7 bacterium, *Pseudomonas fluorescens*, Washington State University, 2007.