Diversity and management of common bunt of wheat in the Great Plains

by

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Abstract

Common bunt is a significant disease of wheat caused by the fungal pathogens *Tilletia tritici* and *Tilletia laevis*. Dwarf bunt is a similar disease of wheat caused by *Tilletia controversa* Kühn, a genetically related pathogen that is regulated by many countries. Management of common bunt and dwarf bunt requires different strategies based on the unique timing of infection for each disease. Proper management thus requires diagnosis. The current diagnostic standards for differentiation of the causal agents of common bunt and dwarf bunt rely on differences in morphological characteristics of teliospores and dissimilarities in the environmental requirements for teliospore germination. Key morphological traits of teliospores include teliospore diameter, the presence or absence of reticulations on the spore surface, and the depth of these protrusions. The rate of teliospore germination at different temperatures is considered the most accurate method for the differentiation of *T. tritici* and *T. controversa*; however, this can take many weeks to complete. Here we evaluated the current standards for species differentiation across a large set of reference isolates and isolates collected from grain samples as well as the efficacy of current seed treatments for management. We characterized the isolates based on spore diameter, reticulation depth, and percent germination over time at 18 °C and 5 °C. We then evaluated conventional management recommendations for these isolates, including the efficacy of seed treatment fungicides in small plot trials. *T. controversa* reference isolates used in this study, as a group, had an average reticulation depth of 1.376 µm. This is smaller than previously suggested for this species, as 1.5-3.5 µm is the range reported by Durán et al. (1961). The average reticulation depth for all *T. tritici* reference cultures was 0.754 µm, which aligns with the published reticulation depth range of 0.5-1.5 µm (Durán, 1961). Variability within individual isolates makes these comparisons uncertain. For example, isolate UT0191 had

reticulations with depths ranging from 0.498 µm to 3.435 µm. Isolate UU0215 had reticulation depths spanning from 0.313 µm to 2.245 µm. Isolate MI0080 had the greatest variability in reticulation depth with a range of 0.593 µm to 2.844 µm. This level of variability suggests that diagnoses based on reticulation depth would likely result in false positives. When unknown isolates were compared to reference cultures used in this study, twelve unknown isolates had average reticulation depths within the range observed for *T. controversa*. Out of these twelve, only two (MI0080 and KS0040) had depths similar to those reported in the literature for *T. controversa*. The mean teliospore diameter in this study (6.807-19.563 µm) was lower than that reported in the literature (*T. tritici:* 14-23.5 µm, *T. laevis:* 14-22 µm, *T. controversa:* 19-24 µm (Goates, 1996)) and appeared to provide no discernable distinction between all three reference species. All *T. controversa* reference cultures had no germination for the duration of the 15-day (18 °C) incubation period, followed by at least 5% germination after a minimum of 30 days incubated at 5°C. Nearly all *T. tritici* isolates surpassed 5% germination in only 6 DPI at 18 °C. Overall, *T. laevis* cultures had low viability. Most *T. laevis* isolates achieved 5% germination after 6 DPI. Most notably, reference isolate UU0238 did not achieve 5% germination until 45 DPI (30 days at 5 °C). Results of this study suggest that isolates of *Tilletia* may be present in the Great Plains that germinate like *T. tritici* but have spore morphology similar to *T. controversa* and these teliospores would likely be misdiagnosed based on the current diagnostic protocols. Our findings suggest that three currently marketed fungicide seed treatments are highly effective for controlling at least one of the morphologically ambiguous isolates characterized in this study. These results have important implications for the understanding, diagnosis, and management of these species in the Great Plains.

Table of Contents

List of Figures vi
List of Tables vii
Acknowledgments viii
Dedication x
Chapter 1 - Common Bunt of Wheat: A Literature Review 1
Chapter 2 - Phenotypic Diversity of Common Bunt and Dwarf Bunt Species in the United States 17
Chapter 3 - Management of Common Bunt with Seed Treatments in Kansas 38
Appendix A - Supplementary Material 1

List of Figures

Figure 2-1 Comparison of average teliospore reticulation depth across samples. 29
Figure 2-2 Comparison of average teliospore diameter. 31
Figure 2-3 Cluster dendrogram representing the results of a hierarchical cluster analysis implemented with Ward's minimum variance method. 33
Figure 2-4 Parameter estimate of percent germination from a linear mixed model of the influence of time on percent germination. 34
Figure 2-5 Graphs depicting percent germination over days post inoculation (DPI) for each isolate. 35
Figure 2-6 Box plot comparing the time (in days) to 5% germination between 69 reference isolates and 44 unknown isolates collected from grain samples across the US. 36
Figure 3-1 Soil temperature at and around planting in each season of field experiments. 50
Figure 3-2 Results from field experiment in Manhattan, KS 2021-22 season 51
Figure 3-3 Results from field experiment in Belleville, KS in 2022-23 season. 53
Figure 3-4 Results from field experiment in Manhattan, KS 2022-23 season 55

List of Tables

Table 1 Comparison of average reticulation depth characteristics across reference isolates. 28
Table 2 Comparison of average teliospore diameter characteristics across reference isolates..... 28
Table 3 Probability values from generalized linear mixed model analysis of main effects and interactions of the main effects on incidence, yield (bu/A), and plant height (cm)..... 49

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Dedication

This thesis is dedicated to my beloved grandfather, Angelo N. Shutkas,

In the face of adversity and with remarkable courage, my grandfather has shown me the true meaning of strength and resilience. Throughout my academic journey, he has been a constant source of inspiration, instilling a deep sense of determination and perseverance.

With a heavy heart, I dedicate this thesis to him as he battles lung cancer. Despite the challenges he faces, he continues to display an incredible spirit and unwavering positivity. His constant encouragement and belief in my abilities have propelled me forward, even in the most difficult times.

My grandfather's continuous faith in me has always been a guiding light, and I am forever grateful for his love, wisdom, and reassurance. He has always been the window through which I see my heritage. This thesis stands as a testament to his enduring influence on my life and academic pursuits.

May this dedication serve as a tribute to my remarkable grandfather, a symbol of love, strength, and the power of the human spirit. I dedicate this work to him with the hope that it may inspire others to overcome challenges and pursue their dreams with resolute determination.

Chapter 1 - Common Bunt of Wheat: A Literature Review

According to the United States Department of Agriculture Economic Research Service, wheat is the third highest-ranked U.S. field crop in terms of both acreage and production (Sowell, 2023). In the 2020-2021 marketing year, the United States exported 26.4 million metric tons (MMT) of wheat, valued at over $6.8 billion, accounting for 13% of global wheat exports (Knisley, 2021). The Great Plains, ranging from Texas through Montana, leads the current hard red winter wheat production in the U.S., accounting for approximately 40 percent of total U.S. wheat production. Kansas is the top producer of Hard Red Winter Wheat in the U.S. (Sowell, 2023). Wheat was estimated to account for 18.3% of the global human calorie intake in 2013 (Savary, 2019). Pests and pathogens threaten this primary calorie source. Savary et al. (2019) estimates that the global yield loss for wheat in 2017 due to pathogens and pests was 21.5%. There was an estimated 10.8% (31.8 million bushels) of lost yield in Kansas during the 2020 growing season attributed to plant diseases. Wheat pathogens threaten wheat production globally and have the potential to interfere with, or even entirely disrupt, international wheat trade. In 2020-2021, approximately 0.3% of the total estimated yield loss caused by disease in Kansas was due to bunt and smut diseases (Hollandbeck, 2020).

Common bunt and dwarf bunt are two wheat disease that affect U.S. wheat production and exports. Common bunt is caused by the pathogens *Tilletia tritici* (Bjerk.) Wint. (syn. *T. caries (DC.) Tul. & C. Tul.*) and *Tilletia laevis* Kühn (syn. *T. foetida (Wallr.) Liro*) which are closely related to the pathogen *T. controversa* J.G. Kühn (syn. TCK), the causal agent of dwarf bunt. *T. controversa*, unlike *T. tritici* and *T. laevis*, is a regulated pathogen. Both common bunt and dwarf bunt cause yield and quality loss and result in an unpleasant, fishy odor due to trimethylamine production (Carris, 2006) that is detectable at contamination levels as low as

0.1% by volume (Matanguihan, 2011b). This compound may play a role in inhibiting premature germination within the sori (Kosted, 2002). Hanna et al. (1932) demonstrated that trimethylamine production was significantly higher in *T. laevis* than in *T. tritici* growing in the same wheat variety. The presence of fetid black teliospores can result in discounts or even rejections of wheat at grain elevators.

Common bunt is present everywhere that wheat is grown around the world. However, it is most detrimental in regions with low adoption of seed treatments or organic wheat production systems. In 1979, common bunt accounted for 5-7% of annual yield loss in North Africa and central Asia, where less than 40% of seed was treated due to financial barriers and intermittent distribution (Hoffman, 1982). Dwarf bunt, on the other hand, is geographically limited as germination of teliospores only occurs after an extended period of snow cover (Goates, 2011). The spread of *T. controversa* through the import and export of plant material is of great concern to international wheat markets. This pathogen has been responsible for halting trade with other countries where dwarf bunt has not been previously reported (Trione, 1982). To prevent the unintentional spread of plant pathogens, phytosanitary practices have been established to mitigate the potential for the movement of teliospores from place to place (Peterson, 2009).

History and Economic Impacts of *Tilletia* spp.

According to Gaudet and Menzies (2012), the first mentions of the blackening of wheat can be found in ancient Greek and Roman texts as well as in the Bible. The Roman gods Rubigus and Rubigo were dedicated to the rust and bunt of wheat (Gaudet, 2012). Though originating in antiquity, common bunt continued to result in extensive losses without much understanding of the biology behind the disease until the mid-1700s. In 1750, the Academie Royale des Belles-

Lettres in France announced a scientific competition that would present an award to the most outstanding dissertation on the bunt of wheat. Although he was not a formally trained scientist, Mathieu du Tillet (1726-1791) won the competition. The role of microbes in infectious diseases had not yet been discovered at this time. However, Tillet proposed that the black dust of bunt was responsible for spreading the disease. To test his hypothesis, Tillet created a large-scale experiment with two factors. The first factor was inoculum. Seed was either naturally clean, naturally contaminated, or experimentally inoculated (Egerton, 2008). The second factor was seed treatment, with non-treated seed acting as the control. To treat the seeds, Tillet washed them in either water, cattle urine, lye solution, a lime and salt mixture, or copper sulfate. He found that seed treatments lowered the infection rate (Matanguihan, 2011b). This supported the hypothesis that the yet-to-be identified black powder not only caused the observed symptoms but also indicated that management of this disease was possible. His experiment was the first to demonstrate that a plant disease was caused by a pathogen, though he never established the identity of the black dust (Egerton, 2008).

Long after Tillet's work, wheat production by the late 1800s had spread into the Pacific Northwest and Great Plains of the United States. Wherever wheat seed moved, bunt followed and continued to cause crop loss. In 1890, the crop losses due to bunt in Kansas were estimated between 25-50% (Gaudet, 2012). Between 1900 and 1960, bunt diseases were responsible for most of the devastation of wheat crops in the Pacific North West. Not only did bunt cause yield losses, but with the invention and widespread use of mechanical harvesters, the bunt spores released during the harvesting process would produce a cloud of black dust that would occasionally ignite, destroying farming equipment and injuring workers (Gaudet, 2012). During

this time, the discovery of dwarf bunt in Montana in 1935 led to the delineation of dwarf bunt as a separate species from common bunt in the early 1950s (Liu, 2009).

In September of 1973, China, concerned about the potential for the introduction of *T. controversa*, instituted a 35-year-long embargo on U.S. wheat grown in the Pacific Northwest after a shipment from the U.S. was stopped from unloading due to suggested contamination with *T. controversa* (Mathre, 1996). According to the USDA reports at the time, by 1998, fifteen countries had regulations in place restricting the entry of *T. controversa* -contaminated grain (Gao, 2014a). By 1999, an agreement was reached between the United States and China to lift the embargo and instead establish a stringent spore threshold of 30,000 teliospores or less per 50 grams of grain in wheat shipments destined for export (Wang, 2005, Goates, 2011). Although this agreement allowed for the unloading of U.S. wheat at any port without additional treatment so long as the requirements were met, China only permitted the transfer of U.S. wheat through a single port where the grain had to be cleaned for a variable fee (Sowell, 2023).

Common bunt has continued to be a persistent problem affecting wheat production in Kansas. Although the disease is nearly always present at low levels, there was a reported increase in incidence of common bunt in the 2019-2020 season. This increased incidence was further complicated by grain samples containing teliospores with morphological characteristics similar to *T. controversa*. Preliminary evaluations of morphological characteristics suggested that these teliospores were *T. tritici*, but with deeper than expected reticulations for the species. This uncertainty in identification of the fungi causing bunt disease of wheat emphasizes the need for improved diagnostic tools.

Taxonomy

In 1847, the Tulasne brothers established the genus *Tilletia* naming it after Mathieu du Tillet (Wang, 2005). The genus *Tilletia* (phylum Basidiomycota, subphylum Ustilaginomycotina, class Ustilaginomycetes, subclass Exobasidiomycetidae, order Tilletiales, family Tilletiaceae) currently includes 186 species of grass-specific pathogenic fungi (Sedaghatjoo, 2022) a majority of which produce teliospores in the host ovaries (Castlebury, 2005). *Tilletia* includes four distinct yet closely related species which cause the bunt of wheat; *T. controversa*, *T. tritici*, *T. laevis*, and *T. indica* (Goates, 1996). Karnal bunt (*T. indica*) in the U.S. is confined to Arizona, therefore this thesis focuses on the other three wheat bunt species (USDA, 2023). *T. controversa*, *T. tritici*, and *T. laevis* are so closely related that differentiation, even genetically, is difficult (Sedaghatjoo, 2021a). Due to the extensive similarity between the three species, there is much contention about whether the common bunt and dwarf bunt fungi should be considered separate species. It has been hypothesized that *T. controversa*, *T. tritici*, and *T. laevis* belong to a single lineage (Levy, 2001, Castlebury, 2005, Carris, 2007, Sharifnabi, 2018, Nguyen, 2019). Russell and Mills (1994) evaluated the characteristics of teliospores from seven unidentified strains using the criteria commonly used to differentiate bunt teliospores, including genetic, biochemical, physiologic, and morphologic traits. The suggested speciation of the isolates changed based on which method was used. Russell and Mills indicate that *T. controversa*, *T. laevis*, and *T. tritici* are variants of a single species that arose due to natural genetic variation (1994, 1993). Currently, accurate differentiation of these species remains a challenge. Bao (2010) performed a phylogenetic analysis on Eurasian and North American isolates of *T. controversa* using ITS rDNA, eukaryotic translation elongation factor 1-alpha (EF1-alpha), and the second largest subunit of RNA polymerase II (RPB2) with three anonymous loci (A13, A16 and P18) which suggested that the dwarf bunt pathogen was not genetically distinct from the common bunt pathogens. Sedaghatjoo

et al. (2022) compared the sequences of 4 *T. tritici*, 5 *T. controversa*, and 2 *T. laevis* genomes demonstrating that *T. controversa* does group separately from the common bunt pathogens, which grouped together in that study.

Teliospore Morphology and Physiology

Tilletia teliospores are generally ovoid to globose with a dark brown, thick, tri-layered exterior (Goates, 1996). *T. controversa* teliospores are globose, 19-24 µm in diameter, and light or dark brown. The cells have a net-like, deeply reticulated (1.5-3 µm) exospore and a prominent hyaline mucoid sheath (1.5-5.5 µm) which extends past the reticulations (Durán, 1961, Carris, 1994, Uvarova, 2020, Goates, 1996). Sterile cells can be colorless to light brown without reticulations. *T. tritici* teliospores are spherical, 14-23.5 µm in diameter, and light or dark brown (Durán, 1961, Goates, 1996). Sterile cells of this species are not colorless. All *T. tritici* teliospores have relatively short reticulations (0.5-1.5 µm) compared to *T. controversa*. Authors disagree regarding the presence or absence of a mucoid sheath on teliospores of this species (Russell, 1994, Goates, 1996, Uvarova, 2020). *T. laevis* teliospores are ellipsoidal to spherical, 14-22µm in diameter, and light or dark brown in color(Durán, 1961, Goates, 1996). These cells have a smooth exospore and a thin mucoid sheath (Uvarova, 2020).

Tilletia controversa teliospores require long periods of cold temperatures (below 10 °C) and adequate moisture to germinate, the process taking between 4-8 weeks (Mathre, 1996, Fuentes-Dávila, 2002). Both *T. tritici* and *T. laevis* teliospores do not require cold temperatures and germinate relatively quickly (7-10 days and 3-5 days, respectively) at 18-20 °C (Fuentes-Dávila, 2002, Purdy, 1963, Russell, 1994). Although, germination rate is said to differ between isolates and pathogenic races (Lowther, 1950, Goates, 1996).

Epidemiology

Both common bunt species have been found to infect a limited number of other members of Poaceae besides wheat, including *Aegilops, Agropyron, Agrostus, Alopecurus, Arrhenatherum, Beckmannia, Bromus, Dactylis, Elymus, Festuca, Holcus, Hordeum, Koeleria, Lolium, Poa, Secale, and Trisetum and X Triticosecale* (Goates, 1996). However, most of these grasses were artificially inoculated. The natural occurrence of disease in these other hosts is not well documented. Carris and Gray (1994) suggest that under axenic conditions, it was shown that *Tilletia fusca*, a species that causes disease in native grasses, could hybridize with *T. tritici*, *T. laevis*, and *T. controversa*.

While common bunt is present on almost every continent, dwarf bunt is confined to parts of the United States, many countries surrounding the Mediterranean, and much of Western Europe (Goates, 1996). These distribution patterns are due to the highly specific climatic conditions required for infection by dwarf bunt. Disease development is contingent on teliospore germination, which requires high moisture and 3 to 8 weeks of -2 to 10 ºC soil temperatures (Mathre, 1996, Fuentes-Dávila, 2002). These specialized conditions are commonly associated with at least 60 days of continuous snow cover (Trione, 1986, Purdy, 1963, Fischer, 1957, Hoffman, 1982). The continuous snow cover helps maintain even temperature and moisture to promote spore germination (Trione, 1986, Peterson, 2009, Baylis, 1958, Grey, 1986).

Modeling Dwarf Bunt Introduction and Establishment

Based on the specific germination requirements established for *T. controversa*, many studies have been performed to evaluate the potential for the introduction and establishment of dwarf bunt in countries where the disease has not been previously reported. In a study conducted by Trione and Hall (1986), snow cover in wheat-growing regions of China over a period of 16

years (1966-1982) was estimated based on data collected from ESSA 3, ITOS 1, ESSA 8, ESSA 9, NOAA 2, NOAA 5, TIROS-N, NOAA 6, and NOAA 7 satellites. The resulting data indicated that none of the 11 instances of extended snow cover in those areas lasted longer than 30 days. Wei et al. (1995) established areas of varying levels of risk in China using a Bio-Climactic Analogical Distance model, which reported the presence of wheat growing areas with high risk and very high risk. The authors also challenged the idea that *T. controversa* required snow cover. Chen et al. (2002) used simulation models in conjunction with geographical information system to predict areas with varying levels of risk. Using this model, Chen et al. (2002) suggested that 19.3% of the winter wheat growing regions in China were considered high to medium risk. It is important to note that Chen et. al. estimated soil moisture at 2 cm depth using soil moisture data from 0-5 cm depth to create this model instead of relying on 0.2 mm of precipitation to represent 60-80% soil surface moisture as was used by the USDA in 1998. Zhou et al. (2006) relied on geophytopathological models in combination with meteorological data over a 50-year time period to calculate the probability of *T. controversa* establishment in China wheat growing regions. Using this particular model, Zhou et al. (2006) reported that 26.99% of winter wheat growing regions were at high risk, followed by 27.32% at medium risk. In 2009, Peterson et al. (2009) created a quantitative risk model for the United States. This quantitative approach essentially followed teliospores from the field to the port to quantify viable inoculum for establishment in a destination country. Based on this comprehensive estimation of inoculum in combination with two separate geophytopathological models evaluating when 42 or more days of precipitation occurred while surface soil temperatures stayed between - 2 and 10 °C during tiller development over a 15-year period, it was demonstrated that 11% of the U.S. winter wheat growing regions were conducive to *T. controversa* introduction. A majority of those conducive

areas were located above 40°N latitude, with the exception of areas in Utah and Colorado. In locations shown in this map to have had disease conducive conditions in 50% or more of the years analyzed, historically significant incidence of dwarf bunt has been reported. It is thought that areas where favorable conditions did not occur as frequently, for example those with 5-24% conducive years, may not be able to support establishment of the disease. While this model did identify spring wheat growing regions in the central northern U.S., *T. controversa* does not infect spring wheat due to temporal distance between timing of teliospore germination and timing of spring wheat emergence (Holton, 1949, Bamberg, 1941, Purdy, 1963, Hoffman, 1982). This model was also used to establish risk in Brazil, Peru, Mexico, and China. In support of the Peterson et al. (2009) model, Goates et al. (2011) conducted field experiments in Kansas (due to the climactic similarity to northern winter wheat growing regions of China). Goates (2011) used a single soil inoculation in conjunction with a highly susceptible cultivar and monitored the location for 4-6 years while reintroducing any resulting disease back into the soil. Results of this study indicated that establishment was highly unlikely even with a susceptible host and high levels of inoculum.

Infection and Disease Cycle

Bunt symptoms include plant stunting, teliospore-filled sori, and occasional "flecking" or yellow spotting on immature wheat leaves (Hoffman, 1982). Common bunt is believed to be a seed-borne disease, while dwarf bunt is seed-borne in respect to transmission to new locations in sori and soil-borne in respect to how seed becomes infected. Wheat infected with bunt produces kernels filled with teliospores (sori). These sori are commonly known as bunt balls. Bunt balls are easily fractured during the harvesting process coating the healthy seed in spores. For common bunt, spore-coated seeds are planted, the teliospores germinate, and the promycelium

forms primary sporidia. These primary sporidia can fuse to form dikaryotic hyphae that infect the pericarp of the wheat seed and penetrate the coleoptile of the germinating seedling. The hyphae establish in the developing wheat head, which will be the site of future teliospore production (Eibel, 2005).

The nuclear cycle begins just before germination. The diploid teliospores undergo meiosis before germination. Haploid nuclei then pass into the promycelium after germination. Each primary sporidium receives a single haploid nucleus which undergoes mitosis. Pathogenic dikaryophase begins following the fusion of opposite mating type primary sporidia. These fused sporidia are referred to as H-bodies due to the H-shape formed by the conjugation peg. Binucleate, dikaryotic secondary sporidia are produced by these H-bodies. The dikaryotic nature is maintained until karyogamy occurs and teliosporogenesis begins (Goates, 1996).

Management Practices

Chemical control with seed treatments has proven most effective in controlling soil and seed-borne inoculum. The active ingredients thiabendazole, bitertanol, etaconazole, hexachlorobenzene, triadimefon, triadimenol, benomyl, chloroneb, fuberidazole, maneb, pyrocarbolid, 2-(Thiocyanomethylthio)-benzothiazole (TCMTB), myclobutanil, carboxin, and pentachloronitrobenzene have all been used in the past for both common bunt and dwarf bunt control, but have since been replaced by new chemistries due to cost, phytotoxicity, or variable efficacy in some environments (Goates, 1996). Current seed treatments for common bunt (not labeled for prevention of dwarf bunt) include, but are not limited to: Relenya® (Mefentrifluconazole Group 3), and Stamina® F4 Cereals (fluxapyroxad Group 7, pyraclostrobin Group 11, triticonazole Group 3, metalaxyl Group 4) from BASF, CruiserMaxx® Vibrance®

Cereals (Sedaxane, Difenoconazole (Group 3), Mefenoxam (Group 4), Thiamethoxam (Group 7)) and Dividend Extreme® (Difenoconazole (Group 3) and Mefenoxam (Group 4)) from Syngenta, and Raxil® Pro MD from Bayer Crop Science (Prothioconazole (Group 3), Tebuconazole (Group 3), and Metalaxyl (Group 4)). Seed treatments are designed to be effective against both soil and seed-borne inoculum. Seed treatment chemistries intended for the prevention of dwarf bunt rely on difenoconazole, which has been shown to manage dwarf bunt effectively. In 1990, difenoconazole was registered for control of soilborne inoculum due to demonstrated effectiveness for control of dwarf bunt in winter wheat (Fushtey, 1961, Sitton, 1993, Buchholz, 1989). Difenoconazole is likely more effective for dwarf bunt prevention because dwarf bunt has an extended lag period before germination, requiring the fungicide to have prolonged activity even at low application rates (Fuentes-Dávila, 2002).

Many organic seed treatments exist, but the modes of action still need to be evaluated. Tillecur®, a yellow mustard powder seed treatment, is one product used commonly in Europe to control *T. tritici* (Waldow, 2007). Many physical spore load reduction methods are used, such as sonication and hot water washing (Matanguihan, 2011b). Many studies have also demonstrated the efficacy of powdered milk in the control of both *T. laevis* and *T. tritici (El-naimi, 2000, Borgen, 2001).*

Host resistance has historically been an important part of bunt management, though effective seed treatment fungicides reduced interest in cultivar development. With recent increases in organic production systems, increased efforts are underway to produce regionally adapted resistant cultivars (Matanguihan, 2011b). Wheat host plant resistance for all three pathogens are controlled by a set of 16 resistance genes (*Bt1-15* and *Btp*), which interact in a gene-for-gene manner (Goates, 2012). This gene-for-gene interaction means host resistance is

conferred when gene products of plant "R" genes interact directly or indirectly with *Avr* gene products produced by the pathogen (typically effectors used in modifying host plant defenses). A single set of 15 differential cultivars are currently used worldwide to determine pathogen races with the addition of a differential containing *Btp* in the United States (Goates, 2012). Goates et al. suggest that vertical resistance (sometimes called race-specific resistance or major gene resistance) is present in this system, meaning disease control for a pathogenic race can be achieved with a single resistance gene in the host (2012). In 2016, according to Chen et al., there were 15 *T. laevis* races, 36 *T. tritici* races, and 19 *T. controversa* races, all of which are differentiated based on reactions to 14 wheat differential lines containing *Bt* genes *Bt1-Bt13*, and *Btp* (2016). All modern Kansas-adapted production wheat varieties are presumed to be susceptible to common bunt and dwarf bunt. In Central Asia and the Caucasus region, more than 200 wheat varieties are highly resistant to the regional races of common bunt. Few, if any, of these varieties are used in production (Madenova, 2021).

Cultural Practices, such as deep sowing (dwarf bunt only, increases the incidence of common bunt by allowing more time for infection to occur), early planting of winter wheat while soil temperatures are above 12 °C, and equipment sanitation, may help reduce the risk of infection but do not provide complete protection. Use of certified seed is a useful non-chemical prevention option in fields where disease has not been detected.

Culturing and Inoculation

Considerable research efforts have been dedicated to culturing and inoculation of bunt fungi. Growth media for common bunt and dwarf bunt include potato sucrose agar and broth, as well as T-19 media (Trione, 1964). T-19 media is a chemically defined medium. Other

germination media includes many complex media types such as: water agar, soil extract agar, and several wheat-based nutrient agars. Soil extract agar is predominantly used for *T. controversa* rather than water or nutrient agar. Boyd and Carris (1998) established that the addition of 1% activated charcoal to the standard 2% water agar resulted in significantly reduced latency periods prior to germination as well as increased overall germination for all taxa tested with the exclusion of *T. tritici* (Boyd, 1998).

Before germination, it is common practice to include a surface sterilization step to reduce contamination. In a study performed by Bonde et al. (1999), it was shown that acidic electrolyzed water (AEW) was an effective sterilization method with longer treatment times that resulted in increased teliospore germination rates when compared to the standard 0.4% NaOCl sterilization treatment.

To inoculate seed with common bunt, approximately 1 g of teliospores per 100 g of healthy seed is gently mixed as described in Goates et al. (1996). Soil inoculation is reserved for dwarf bunt. Dwarf bunt seed inoculation requires germinating teliospores before mixing them with seed (Goates, 1996). Inoculated trays are then covered with aluminum foil and vernalized for 3 weeks for spring wheat and 8 weeks for winter wheat varieties.

Fernandez and Durán (1978) infected susceptible wheat cultivars during the flag leaf stage via hypodermic injection of germinating teliospores. This pioneering work has been replicated with variable success by many research teams since. Churchill and Mills (1985) used this technique to evaluate the segregation of genetic markers by injecting sexually compatible secondary sporidia. Trail (1984) also used this technique with haploid secondary sporidia instead of germinated teliospores.

Diagnostic Techniques

Spore Morphology- USDA Protocol

The Federal Grain Inspection Service (FGIS) currently uses a protocol developed by the USDA-ARS, and agreed upon by China in the 1999 agricultural trade agreement, to determine the identity and quantity of *Tilletia* spores in grain samples originating from the U.S. destined for export (Peterson, 2021). This protocol relies on the established differences in teliospore reticulation depth between *T. controversa* and *T. tritici*. According to this protocol, a 50g sample of grain is washed using a tween solution to extract any teliospores on the grain surface. The number and reticulation depth of teliospores present in the suspension are used to determine species. Unfortunately, this morphological identification cannot yet be confirmed by molecular techniques and is prone to error due to overlapping distribution of reticulation depth between the two reticulated species.

Molecular Techniques

All three species are genetically similar, sharing greater than 99% average nucleotide identity (Sedaghatjoo, 2021a), which has made developing a universal molecular diagnostic technique challenging. The development of a molecular identification assay for the diagnosis of *T. controversa* has been the goal of several research teams. Despite significant effort, there is still no widely used procedure for molecular identification. Some of the approaches evaluated as possible diagnostic tools are described here. Pimentel et al. (1998) used restriction fragment length polymorphism (RFLP) markers in 1998 and random amplified polymorphism DNA in 2000 (Pimentel, 2000). Neither technique was able to distinguish between the three closely related species. McDonald et al. (2000) used repetitive-sequence-based polymerase chain reaction (rep-PCR) fingerprinting. PCR primers were developed by Kochanova et al. in 2004 (Kochanová, 2004). In 2005, Eibel et al. published an evaluation of the primer pair

Tcar2A/*Tcar2B* for use in polymerase chain reaction detection of the bunt pathogen *T. tritici* in shoots, leaves, and seedlings, as well as an immunological detection via a double antibody sandwich enzyme-linked immunosorbent assay intending to develop early, reliable, and specific detection methods for use in resistance breeding (Eibel, 2005). They determined that the primer set was unsuccessful at differentiating *T. tritici* from *T. controversa,* and the ELISA was ineffective at detecting teliospores or spore extracts, meaning the primer set was unfit for species-level diagnosis. Cross-reactivity with *T. laevis* was not tested. The ELISA demonstrated that fungal concentration was variable between tissues, with extracts from seeds and seedling roots providing the highest signals (Eibel, 2005). Liu et al.(2009) developed SCAR markers using AFLP. Yuan et al. (2009) employed RAPD primer-mediated asymmetric-PCR (RM-PCR) to select *T. controversa*-specific markers from 18 *T. controversa* strains and 29 *T. tritici* strains. They discovered a 1,322 bp DNA fragment (PR32) in *T. controversa* which showed no homology to *T. tritici* isolates or other fungal species used for validation. This fragment was used to develop conventional PCR primers ($CQUTCK_2$/$CQUTCK_3$). Visualization of the PCR product resulted in a 747 bp *T. controversa*-specific band. Gao et al. (2014b) used ISSR to identify *T. controversa*-specific molecular markers from 19 *T. controversa* isolates, 13 *T. laevis* isolates, and 24 *T. tritici* isolates. They discovered a 678 bp *T. controversa*-specific DNA fragment which they used to develop sequence-characterized amplified region (SCAR) primers. These primers amplified a 372 bp *T. controversa*-specific DNA fragment. In 2019, Nguyen et al. generated whole genome data for multiple strains of *T. controversa* and the common bunt pathogens. These genome sequences identified species-specific genes (Nguyen, 2019). From these sequences, a single *T. controversa*-specific primer was developed, which amplified a 120 bp amplicon (Nguyen, 2019). Sedaghatjoo et al. (2021a) also used whole genome sequences from 21 genomes

from 6 different *Tilletia* species to identify *T. controversa*-specific sequences. Loop-mediated isothermal amplification LAMP primers were developed for one candidate region, which was tested against 11 *Tilletia* species, including 39 specimens of *T. controversa,* 92 specimens of *T. tritici*, and 40 specimens of *T. laevis*. Primers O_8_2F3 and O_8_2B3 used in this assay resulted in PCR products 209 bp in length. This assay did have cross-reactivity with *T. trabutii* (Sedaghatjoo, 2021b). Despite the efforts of these research teams, regrettably, there is still no diagnostic primer set for consistent, highly specific, and accurate identification of *T. controversa*.

Research Objectives

With the future of U.S. wheat export depending on clean wheat production, it is of the highest importance that easy and definitive diagnostic methods be developed to prevent erroneous diagnoses and that effective management practices for growers be established to control the occurrence of these diseases. The goals of this project were to document the phenotypic diversity of the causal agents of common bunt, evaluate current diagnostic techniques, and establish clear management practices to reduce the incidence of common bunt

Chapter 2 - Phenotypic Diversity of Common Bunt and Dwarf Bunt Species in the United States

Common bunt (syn. stinking smut), caused by the pathogens *Tilletia laevis* and *Tilletia tritici*, is a disease predominately affecting winter wheat. Infection occurs when teliospores on the seed surface from the previous season's harvest germinate after planting as the seedling emerges from the pericarp. As infected heads reach maturity, the hyphae produce teliospores that replace the starch in mature grain with teliospores that have a fishlike odor, resulting in yield and quality loss as well as discounts or even rejection at the elevator (Peterson, 2009). Dwarf bunt, caused by *T. controversa*, is geographically isolated, unlike common bunt, due to unique climatic requirements and is therefore regulated to prevent the spread to new locations (Goates, 1996, Mathre, 1996, Trione, 1982, Trione, 1986, Peterson *et al.*, 2009, Goates *et al.*, 2011).

Common bunt is a persistent problem affecting wheat production in Kansas. Although the disease is reported in many years, there was an increase in incidence of common bunt in 2020. This increased incidence was further complicated by grain samples containing teliospores with morphological characteristics similar to *T. controversa*. Preliminary findings suggested that these teliospores were *T. tritici*, but with deeper than expected reticulations for the species. This uncertainty in identification of the fungi causing bunt disease of wheat emphasizes the need for improved diagnostic tools.

Because *T. controversa* has a limited global distribution, there have been import regulations imposed by some countries to limit the risk of accidental introductions (Goates, 1996, Mathre, 1996, Trione, 1982, Trione, 1986, Peterson et al., 2009, Goates et al., 2011). In 1973, after rejecting a shipment of grain due to the presence of *T. controversa* spores, China instituted an embargo on all wheat originating from the Pacific Northwest region of the United

States (Mathre, 1996, Peterson et al., 2009, Trione, 1982). Following the lead of China, as many as fifteen countries implemented a ban on *T. controversa*-contaminated grain (Peterson, 2009). Some countries opted for a spore threshold instead of an outright ban. This approach had been used to regulate grain imports in Germany, Austria, Switzerland, Scotland, Denmark, and the United Kingdom (Matanguihan, 2011b). By 1999, after extensive communication and negotiation between the US and China, an agreement was reached to implement a stringent threshold for the concentration of spores in grain shipments. Under the 1999 agricultural trade agreement, current laws regulating the import of U.S. wheat by China allow for a maximum of 30,000 teliospores of *T. controversa* per 50 g of grain. Because of the importance of this export market for the United States, proper differentiation of these species is critical. Teliospores are currently diagnosed microscopically in the U.S. based on differences in cell wall morphology.

T. laevis, *T. tritici*, and *T. controversa* differ in teliospore morphology and temperature requirements for germination (Goates, 1996, Mathre, 1996, Russell, 1994, Pascoe, 2006). Both teliospore diameter and reticulation depth differ between the three species. Teliospore diameters range from 14-22 µm for *T. laevis*, 14-23.5 µm for *T. tritici*, and 19-24 µm for *T. controversa* (Fernandez, 1978, Goates, 1996). As for reticulation depth, *T. laevis* has an entirely smooth teliospore wall and is easily distinguished from the other species because *T. tritici* and *T. controversa* have reticulated spore walls. The reticulation depths of *T. tritici* range between 0.5-1.5µm and *T. controversa* reticulations range between 1.5-3µm (Durán, 1961, Goates, 1996). The USDA has adopted this subtle difference as the standard for diagnosing these species in a grain export environment where rapid identification is critical (Peterson, 2009). However, as previously suggested, there is a high degree of overlap between the species and even high variation between isolates of the same species (Personal Comm. Blair Goates). Due to this

overlap, the potential exists for false positive and false negative diagnostic results in grain export environments.

Germination requirements are the gold standard for distinguishing the two species, reflecting the key life history differences between these fungi. Teliospores of *T. tritici* and *T. laevis* germinate within 3-5 days at 18 °C, while *T. controversa* teliospores will germinate at 5 °C in 3-6 weeks with supplemental lighting. (Fuentes-Dávila, 2002). These temperatures reflect the infection timing of the winter wheat crop. The pathogens that cause common bunt infect rapidly after the wheat plant germinates in soils with moderate temperatures, while *T. controversa* requires a long dormancy period in cold soils. In theory, germinating teliospores of this pathogen should accurately distinguish *T. tritici* from *T. controversa*. Unfortunately, the germination process can take upwards of 6 weeks, making this method ill-suited for rapid or high-throughput diagnosis. Additionally, loss of spore viability in shipment may reduce the efficacy of a diagnostic method relying on live spore germination.

Without definitive diagnosis, critical questions exist about isolates of these species that were detected in Kansas post-2000. The objective of the present study was to characterize the variability of phenotypic traits among isolates recently collected from Kansas, Michigan, and the Pacific Northwest, as well as a culture collection from the USDA-ARS National Small Grains Germplasm Research Facility.

Methods and Materials

Origin and Maintenance of Cultures

In total, 102 *Tilletia* samples were evaluated in this study (Table A- 1), including 69 reference cultures consisting of *T. tritici*, (UU0195-UU0229) *T. laevis* (UU0230-UU0234,

UU0236-UU0239, UU0245-UU0250), and *T. controversa* (UT0175-UU0193) that were obtained from the USDA-ARS National Small Grains Germplasm Research Facility. These reference cultures were previously characterized and deposited by Dr. Blair Goates and were received by our group as bunt balls containing teliospores. In addition to reference cultures, recently collected samples were obtained from Kansas, Oklahoma, Ohio, Oregon, Utah, Washington, Montana, Idaho, and Michigan. Recently collected samples were maintained in individual double-bagged plastic storage bags at room temperature, either as infested grain or intact bunt balls, until single bunt balls were chosen from each sample. Teliospores can remain viable at room temperature for long periods of time. The reference samples were maintained in cryovials at -20°C.

A single, intact bunt ball was selected from each grain sample. Its contents were divided into four aliquots for subsequent morphological analysis, three experimental repetitions of germination analysis, and a final portion was saved to create a purified culture collection.

Morphological Analysis

Teliospore measurements. The subset of spores from each sample designated for spore measurements were suspended in Shear's mounting fluid (Goates, 1996) and examined via light microscopy. One-hundred teliospores were analyzed per sample using a Plan acromat fluorite 100x magnification oil immersion objective connected to an Olympus BX50 upright light microscope. Two diameter measurements were taken on each spore. For reticulated samples, the depth of four reticulations were measured for each of the 100 teliospores in addition to the two spore diameter measurements. Reticulation measurements were achieved using an Accu-Scope Excelis™ HD 1080p HDMI microscope camera in conjunction with the CaptaVision+ imaging

program attached to the scope with an Olympus microscope U-SPT camera tube adapter. The imaging program was calibrated using a stage micrometer calibration slide. Reticulations were measured at the spore hemisphere by adjusting the condenser aperture diaphragm until only the center plane was visible, then measuring from the outside of the exospore wall to the tip of the spike. Teliospore diameters were taken at the same time and measured from one side of the cell wall to the other across the midpoint not including the reticulations or mucoid sheath if present.

Germination Analysis. Using a subset of teliospores from the same bunt ball used for reticulation measurements, teliospores were surface sterilized using a 0.4% sodium hypochlorite (NaClO) solution for 1 minute, then rinsed three times with sterile ddH2O to remove any residual NaClO. One-hundred microliters of the sanitized teliospore suspension were then spread on T-19 media using a sterile glass plate spreader and left to dry in a biosafety cabinet until the liquid was absorbed into the agar preventing accidental movement of the teliospores. T-19 media was prepared as described by Trione (1964). T-19 is a chemically defined medium developed to isolate *Tilletia* species from infected plant tissue. After spores were plated, plates were inverted and incubated at 18 °C in a Percival model I-30NL incubator with 10 hours of continuous white light provided by a supplemental LED lamp. One hundred individual teliospores were identified and monitored throughout the experiment. The teliospores were assessed for germination at 3, 6, 9, 12, and 15 days post inoculation or until 30% germination was achieved. Plates that did not achieve 30% germination within the 15-day timeframe were moved to 5 °C with 10 hours of continuous supplemental light and were observed at 30, 45, and 60 days post temperature change. Spores were considered germinated when the promycelium was at least the same size as the diameter of the teliospore or primary sporidia were present (whichever occurred first).

Data Analysis

An analysis of variance (ANOVA) procedure was used as a preliminary assessment of differences in mean reticulation depths and diameter measurements among isolates with the *aov()* function in the base statistical package within the R programming environment (R Core Team, 2023). Tukey's honest significant difference (HSD) was used to facilitate sample mean separation and present the estimated marginal mean from the ANOVA. The r package *emmeans* (R, 2023) facilitated visualization of means comparisons. To further explore groupings of samples based on reticulation depth and spore diameter, a hierarchical cluster analysis was implemented with Ward's minimum variance method utilizing the *hclust()* function in R. Time to germination was analyzed with a mixed model analysis of variance for a split plot design with repeated measures using the MIXED procedure in SAS v. 9.4.

Results

Reticulation depth analysis

The mean reticulation depth across all *T. controversa* reference cultures was 1.376 µm (Table 1, Figure 2-1), notably lower than the previously published standards for the species. Nearly all individual *T. controversa* reference cultures had mean reticulation depths between 1-1.5 µm with the exception of ID0178, WA0186, UT0191, and UU0038, which had averages of 1.584 µm, 1.534 µm, 1.860 µm, and 1.723 µm, respectively. The maximum reticulation depth observed for the *T. controversa* reference cultures belonged to UU0038 and was 3.56 µm deep. The minimum reticulation depth which belonged to ID0183, was 0.175 µm deep. The isolate with the most extensive range of reticulation depths was UT0191 which spanned from 0.498 µm to 3.435 µm.

The average reticulation depth for all *T. tritici* reference cultures was 0.754 µm (Table 1), with mean reticulation depths for individual *T. tritici* reference cultures falling between 0.163-0.998 µm (Table 1). The maximum reticulation depth observed for the *T. tritici* reference cultures was in UU0215, which had an individual spore with an average reticulation depth of 2.245 µm. The minimum reticulation depth belonging to UU0204 was 0.107 µm deep. The most variable *T. tritici* reference isolate was also UU0215, with reticulation depths spanning from 0.313 µm to 2.245 µm.

When comparing mean reticulation depth across species the sample means were significantly different ($P = < 0.00001$). This is not surprising given the wide range across the samples (Table 1, Figure 2-1). The mean reticulation depths for all unknown isolates were interspersed between both reticulated reference species creating a continuum of reticulation depths rather than a stark contrast between *T. controversa*-like isolates and *T. tritici*-like isolates (Figure 2-1). Nearly all of the reticulated unknown samples evaluated had average reticulation depths similar to those described for *T. tritici* despite individual reticulations far exceeding the suggested upper limit. Although, unknown isolates KS0019, KS0024, KS0040, MI0076, MI0078, MI0080, and UT0168 grouped more closely with the *T. controversa* reference isolates, with mean reticulation depths falling above 1 µm (Figure 2-1). Isolates KS0016, KS0018, KS0020, KS0027, OK0029, OK0030, KS0032-KS0035 and OR0169 also grouped with *T. tritici* reference cultures. The minimum reticulation depth of any unknown isolate belonged to isolate KS0019 with a depth of 0.121 µm. The maximum reticulation depth observed in the unknown isolates belonged to isolate MI0080, which also had the greatest variability in reticulation depth with a range of 0.593 µm to 2.844 µm. Unknown isolates KS0001-KS0015, KS0021, UU0041-UU0043, UU0087- UU0089, and KS0171 had no discernable surface reticulations.

Teliospore diameter

The mean teliospore diameter in this study was lower than that reported in the literature and the variation among evaluated isolates was highly significant (P = < 0.00001, Table 2, Figure 2-2). Average diameters for all isolates tested were between 6.807 µm and 19.563 µm. The teliospore with the largest diameter (28.287 µm) belonged to UU0228. The smallest diameter (4.772 µm) belonged to KS0031, which did not group with any of the other samples (Figure 2-2). The most extensive range of diameters belonged to KS0033, which spanned from 4.810 µm-17.727 µm. Post-hoc mean comparisons did not group the isolates by species but demonstrated high overlap in the population (Figure 2-2).

Hierarchical cluster analysis was utilized to determine if mean reticulation depths and teliospore diameters could be utilized to group samples according to species (Figure 2-3). Generally, this method characterized the *T. laevis* samples and some of the *T. tritici* samples, but could not distinguish several *T. tritici* samples from reference *T. controversa* samples.

Germination analysis

The main effects (Species, Days Post Inoculation (DPI)) as well as the interactions significantly influenced percent germination (P <0.0001). The *T. tritici* reference isolates had the highest total levels of germination as a group (Figure 2-4, Figure 2-5, Figure 2-6) and generally reached > 50% germination by 15 DPI. The *T. laevis* and unknown isolates, as a group, generally reached ~20% germination by 15 DPI (Figure 2-4, Figure 2-5, Figure 2-6). As expected, most *T. controversa* isolates did not germinate until they were transferred to cold temperatures and had no germination for the duration of the 15-day (18 °C) incubation period, followed by at least 5%

germination after a minimum of 30 days incubated at 5 °C (Figure 2-4, Figure 2-5, Figure 2-6). Michigan isolates MI0076 and MI0078 did not germinate within the 75-day timeframe, however in preliminary studies, these isolates germinated after 6-12 months when incubated at 5 °C with supplemental light. Unknown isolates KS0016, KS0018, and KS0019, previously thought to be *T. tritici* based on reticulation depths and preliminary germination results, did not achieve 5% germination until 45 DPI. The viability of *T. laevis* reference cultures was extremely low overall. Most *T. laevis* isolates did not achieve 5% germination until 6 DPI. Interestingly, reference isolate L9 did not achieve 5% germination until 45 dpi (30 days at 5 °C – Figure 2-4, Figure 2-5, Figure 2-6).

Discussion

All of the *T. controversa* reference cultures used in this study had on average shorter reticulations (Table 1) than previously described in the literature. Previous work has suggested *T. controversa* has reticulation depths between 1.5-3 µm while *T. tritici* has reticulation depths less than 1.5 µm (Goates, 1996, Durán, 1961, Trione, 1982). It could be that these general thresholds were made from too few observations. Original data from these studies were difficult to trace. The highly variable range of reticulation depths in this study suggests that the use of teliospore reticulation depth for the diagnostic identification of species allows for a high probability of misdiagnosis and false positive reports. The use of averages and extremes are often an oversimplification of the variability present in these species and are not recommended for use in diagnostic settings which require precision and accuracy. The most notable shortcoming of relying on a reticulation depth threshold for species identification is, that even with a large

number of reticulations measured, the average may fall below that threshold and result in false negative samples.

Similar to those values reported by Goates (1996) and Durán (1961), teliospore diameter measurements (Table 2) in this study seemed to have a high level of overlap between species. Therefore, this should not be a key component of differentiation. The diameter values in this study were smaller than those reported by Goates (1996) and Durán and Fischer (1961). Whether this was due to differences in sample selection, sample size, or measuring technique is unclear. It should be noted that measurements in this study were made excluding the reticulations, potentially leading to results that conflict with previous literature. A cluster analysis including both diameter and reticulation depth did not distinguish samples by previous species classifications.

Germination, while considered the gold standard for species differentiation, can be an obstacle for high-throughput diagnostics as some isolates may take months to germinate. Additionally, though inconsistencies existed between repetitions, isolates like UU0238, a reference isolate of *T. laevis* which germinated at 45 DPI at 5 ºC, dispute the certainty of this concept and illustrate the importance of utilizing multiple testing methods for identification. The viability of the teliospores in this study was often low making evaluation of germination requirements challenging.

Overall, combining average reticulation depth, teliospore diameter, and time until 5% germination did not clearly diagnose potential species of isolates with intermediate traits. This is due to a high level of variability in these characteristics within a species leading to high levels of overlap between the species. This work demonstrates that future efforts should be aimed at disentangling the identification of these species. This is supported by previous studies that

suggest *T. tritici*, *T. laevis* and *T. controversa* do not represent true independent species (Sedaghatjoo, 2022, Russell, 1994, Russell, 1993). Despite the shortcomings of these techniques, they could be combined to develop of a rough dichotomous diagnostic key to quickly identify samples containing bunt balls with teliospores that have characteristics which fall outside the zones of uncertainty. Such a diagnostic tool would need to undergo rigorous validation before widespread use. This tool could be updated at a later date for molecular confirmation and diagnosis of intermediate samples when standard molecular tests are established.

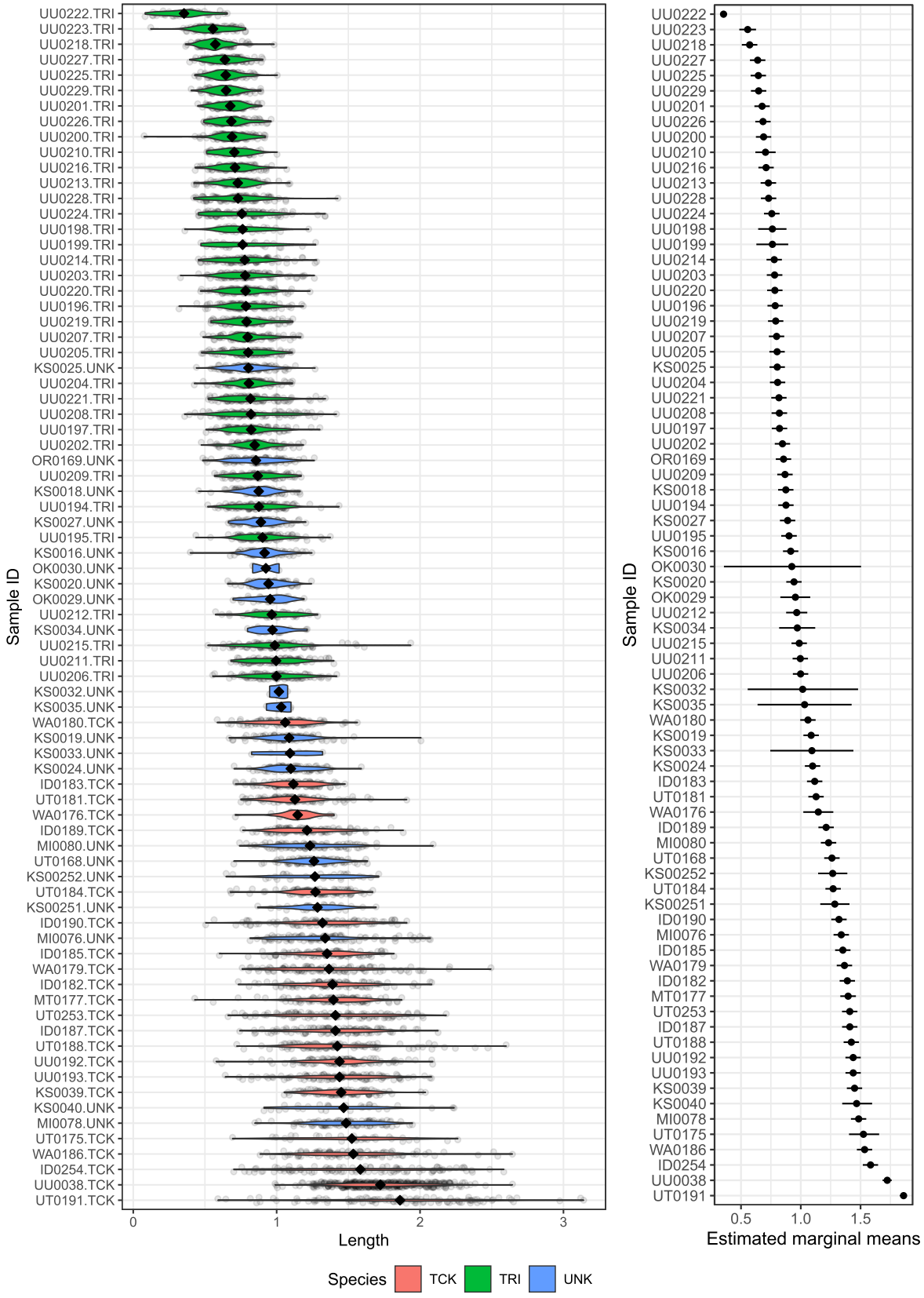
Table 1 Comparison of average reticulation depth characteristics across reference isolates.

Species	Mean Reticulation Depth (µm)	Minimum Average Reticulation Depth (µm)	Maximum Average Reticulation Depth (µm)
T. laevis	NA	NA	NA
T. tritici	0.754	0.163	0.998
T. controversa	1.376	1.060	1.860

Table 2 Comparison of average teliospore diameter characteristics across reference isolates.

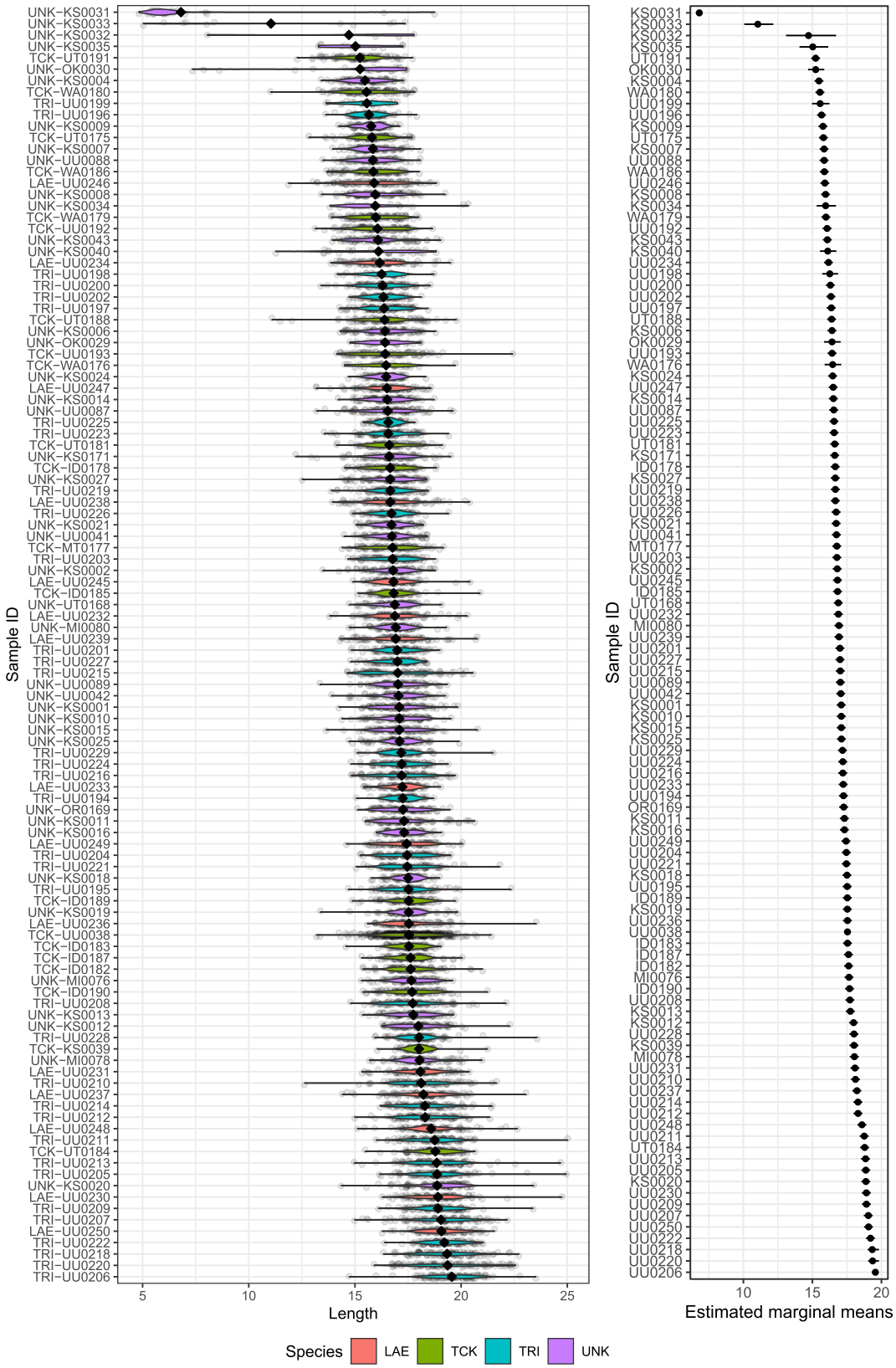
Species	Mean Teliospore Diameter (µm)	Minimum Average Teliospore Diameter (µm)	Maximum Average Teliospore Diameter (µm)
T. laevis	17.397	15.904	19.070
T. tritici	17.557	15.562	19.563
T. controversa	16.708	15.547	18.787

Figure 2-1 Comparison of average teliospore reticulation depth across samples.



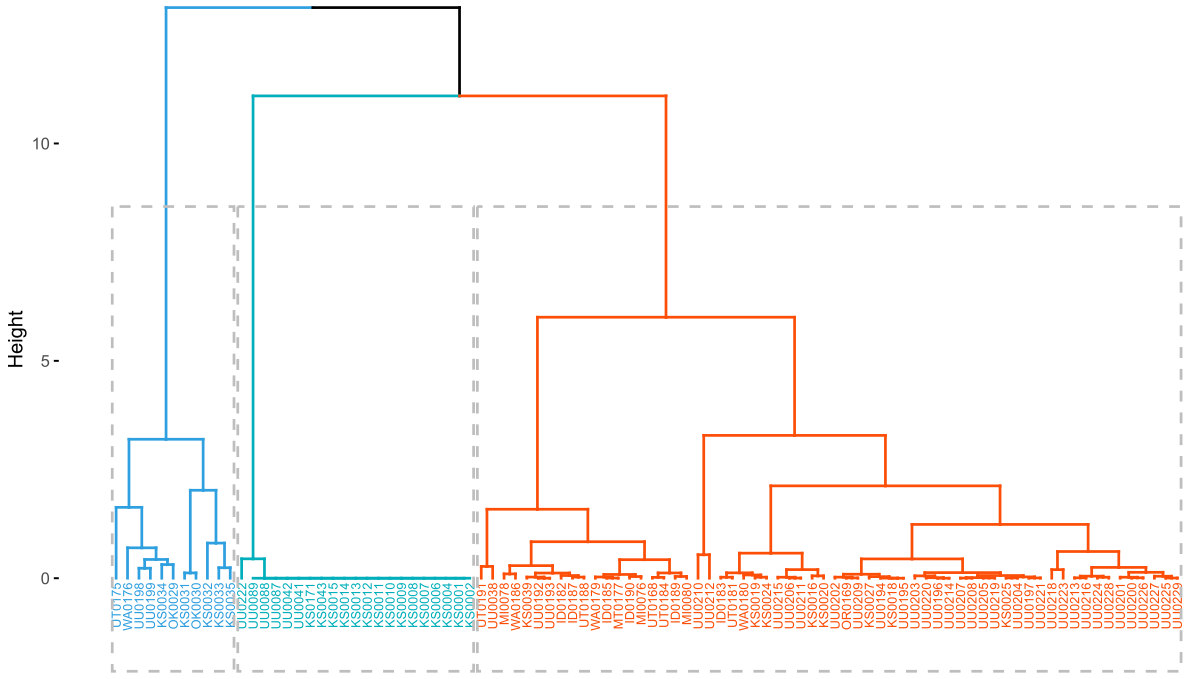
Violin plot of average reticulation depths for each sample (panel on left) compared across 44 unknown samples and 69 reference isolates. The width of the violins represents the density of data points. The reference isolates are shown in pink (TCK) or green (TRI (*T. tritici*)). Unknown isolates (UNK) are shown in blue. The x-axis represents the reticulation depth values, and the y-axis shows the isolate identifiers. The panel on the right depicts the estimated marginal means from our analysis of variance for each sample. Overlapping lines represent samples that were not significantly different according to Tukey's honest significant difference. Samples with no reticulations were omitted from visualization.

Figure 2-2 Comparison of average teliospore diameter.



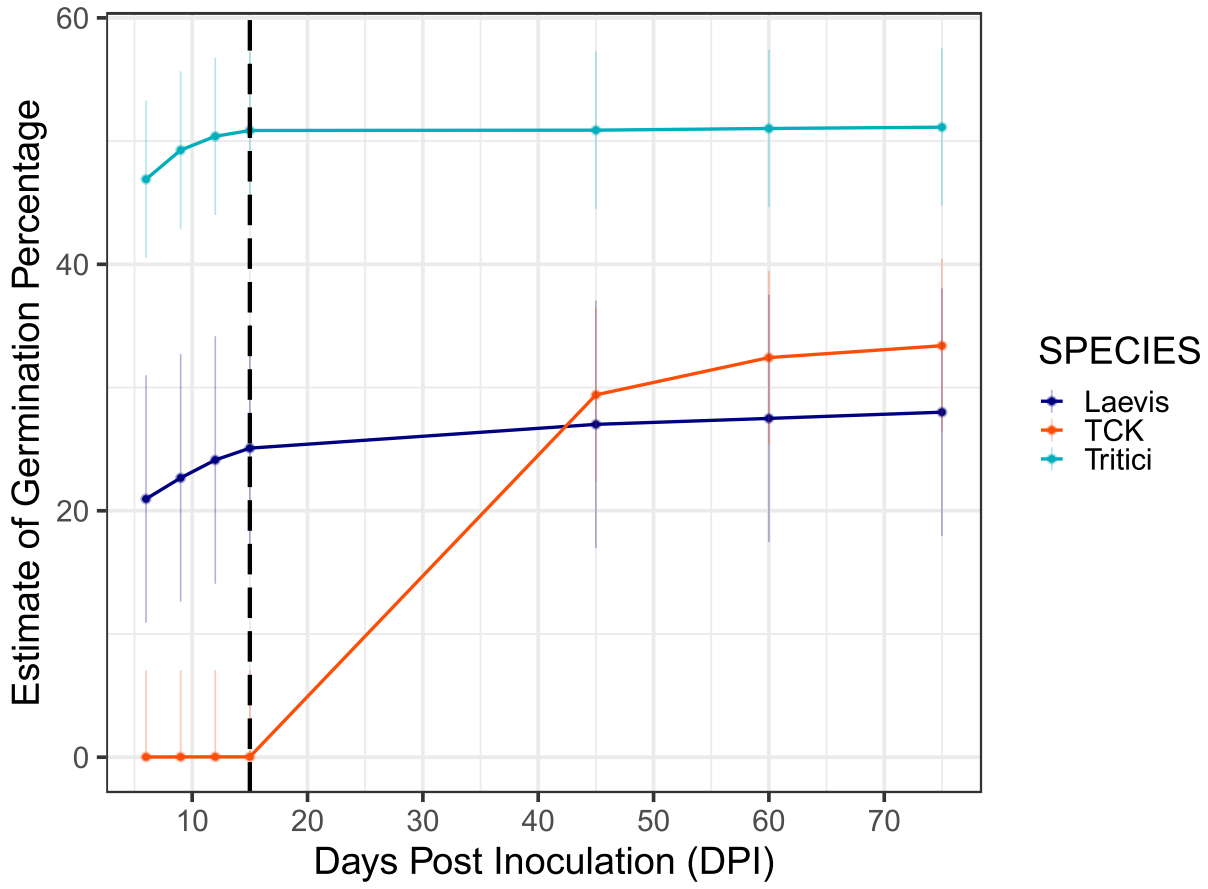
Violin plot of average teliospore diameter (of 100 spores measured per sample) by sample (panel on left) compared across 44 unknown samples and 69 reference isolates. The width of the violins represents the density of data points. The reference isolates are shown in pink (*T. laevis*), blue (*T. tritici*) or green (*T. controversa*). Unknown isolates are shown in purple. The x-axis represents the reticulation depth values, and the y-axis shows the isolate identifiers. The panel on the right depicts the estimated marginal means from our analysis of variance for each sample. Overlapping lines represent samples that were not significant according to Tukey's honest significant difference.

Figure 2-3 Cluster dendrogram representing the results of a hierarchical cluster analysis implemented with Ward's minimum variance method.



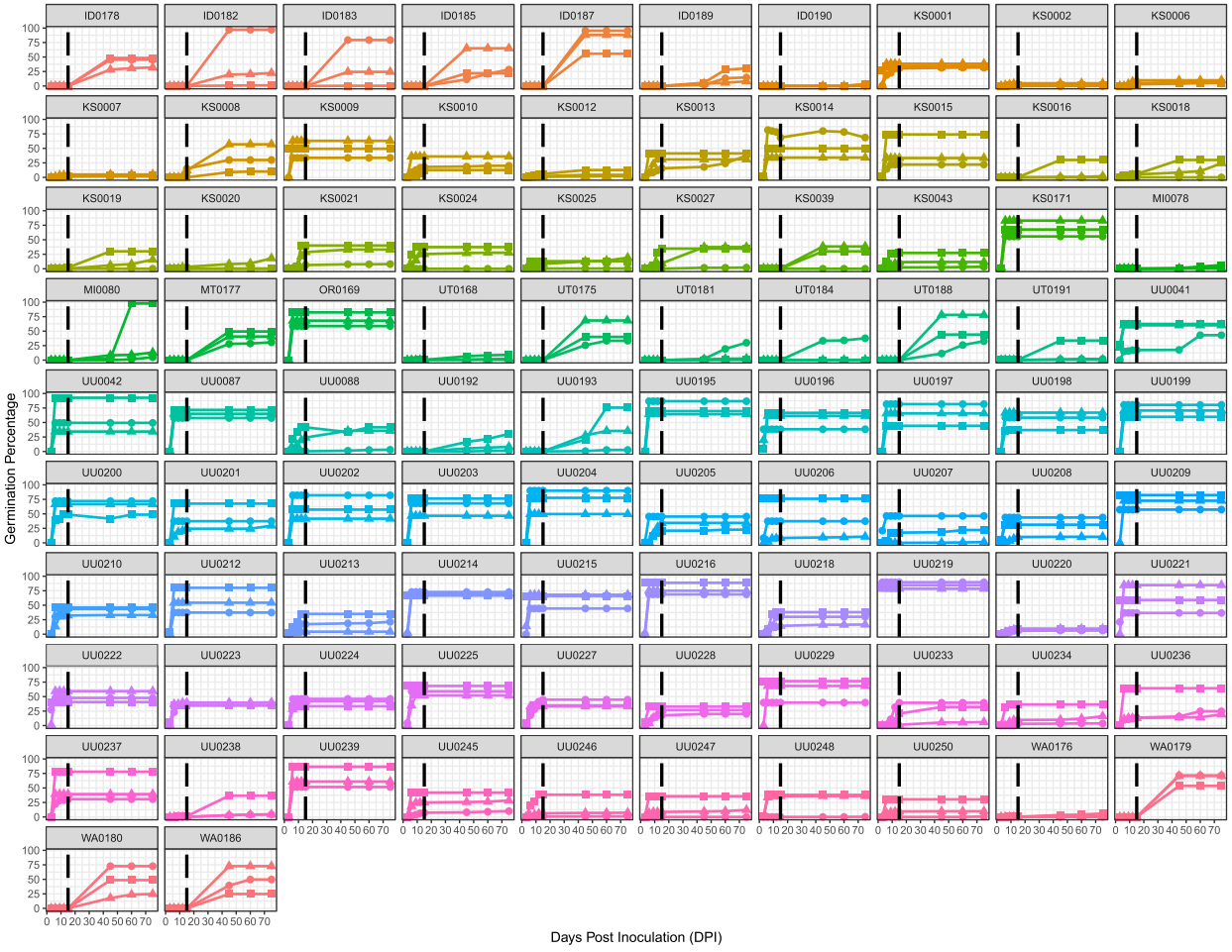
Cluster dendrogram representing the results of a hierarchical cluster analysis implemented with Ward's minimum variance method. Clustering is based on means of reticulation depth and teliospore diameter for each sample. Branches that share the same color fall within the same cluster.

Figure 2-4 Parameter estimate of percent germination from a linear mixed model of the influence of time on percent germination.



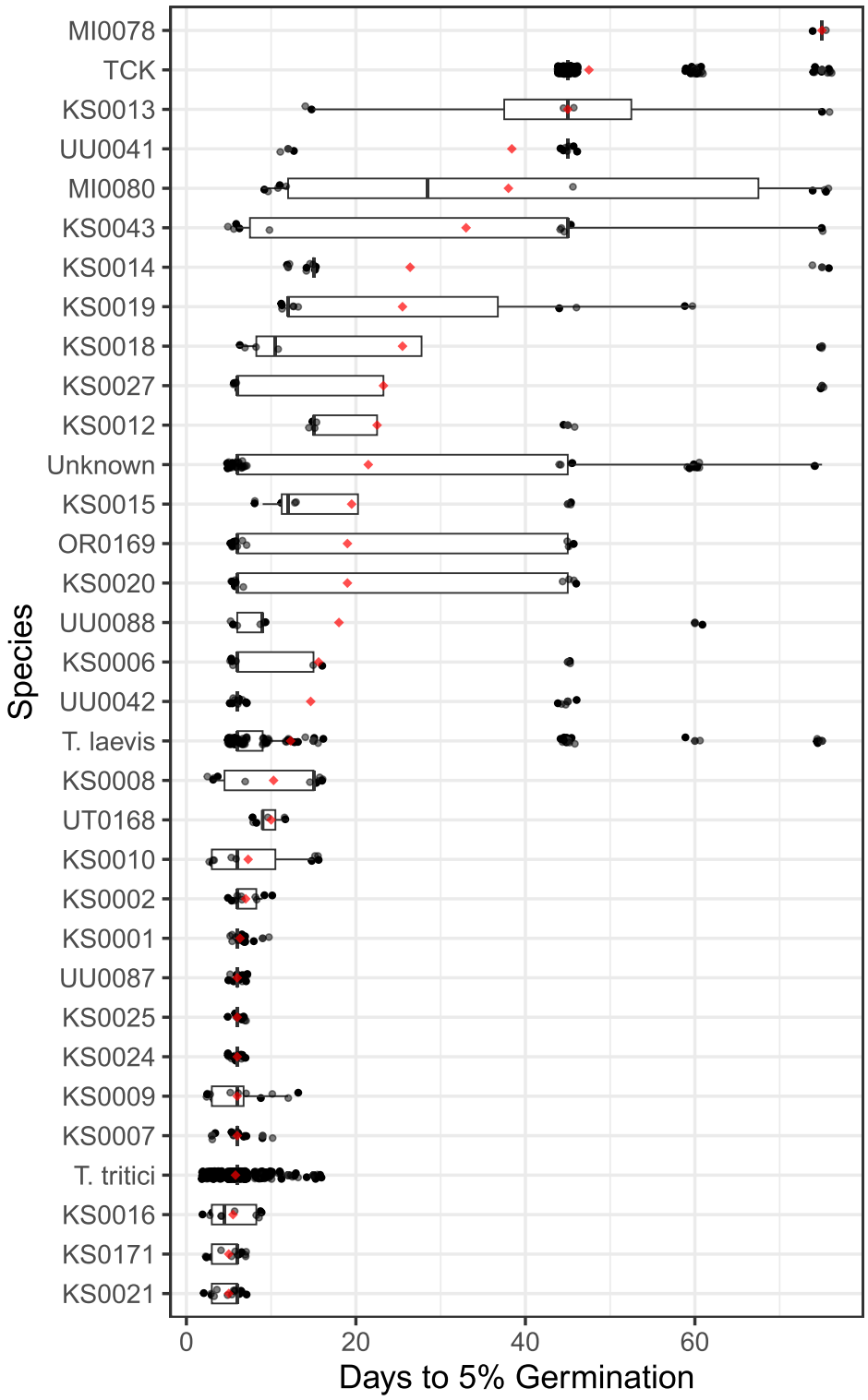
Samples are grouped by species (color). Samples with unknown species were omitted. Vertical lines represent upper and lower confidence intervals for the estimate. Plates that did not achieve 30% germination within the 15-day timeframe were moved to 5 °C so estimates after 15 days reflect germination post-temperature change.

Figure 2-5 Graphs depicting percent germination over days post inoculation (DPI) for each isolate.



Individual graphs illustrate germination percentage over days post inoculation (DPI) for each isolate. Individual lines within each graph illustrate the average germination of three replications performed within each of the three replications shown.

Figure 2-6 Box plot comparing the time (in days) to 5% germination between 69 reference isolates and 44 unknown isolates collected from grain samples across the US.



All reference samples were grouped within species (*T. controversa*, *T. tritici*, *T. laevis*). Replications that failed to germinate were removed from this visualization. Only samples where a minimum number of replicates germinated were included, removing isolates that were assumed nonviable. The box represents the interquartile range (IQR) of the data, with the median indicated by the line inside the box. Red diamonds represent group means.

Chapter 3 - Management of Common Bunt with Seed Treatments in Kansas

Introduction

Common bunt is a significant disease in most wheat-producing regions globally caused by the pathogens *T. tritici* and *T. laevis*. This disease results in yield and quality losses due to the complete replacement of infected grain with foul-smelling teliospores in the form of sori. During harvest, these sori, known as 'bunt balls' break open, releasing teliospores that coat healthy seeds, serving as inoculum for the subsequent season. The pathogen is transmitted via seed contaminated with teliospores from season to season or field to field. Not only does this disease result in yield loss and quality loss, but it also produces a foul, fishlike odor in infected grain due to the synthesis of the organic trimethylamine compound by these fungi (Hanna, 1932). Dwarf bunt is a similar disease of wheat caused by the pathogen *T. controversa*. Although these three *Tilletia spp*. share greater than 99% average nucleotide identity (Sedaghatjoo, 2021a), they have very distinct epidemiological requirements that influence both geographic distribution and management.

Despite 16 resistance genes (*Bt1-Bt15* and *Btp*) having been identified for control of common bunt and dwarf bunt, based on classic gene-for-gene reactions (Goates, 2012, Hoffmann, 1975), in modern conventional agriculture, this disease has been almost exclusively managed with fungicide seed treatments (Matanguihan, 2011a). In recent years, research has found that modern seed treatments are very effective at controlling the disease caused by the pathogens *T. tritici* (Ashley, 2006), *T. controversa* (Sitton, 1993), and *T. laevis* (Johnston, 2005a, Johnston, 2005b, Johnston, 2004, Johnston, 2001b, Johnston, 2001a, Bockus, 2001, Johnston,

1999a, Johnston, 1999b) provided there is adequate coverage of the seed treatment (Johnston, 2005b).

Important differences exist between current management recommendations for common bunt and dwarf bunt. Currently, only products with the active ingredient difenoconazole have been proven to be effective at controlling dwarf bunt (Sitton, 1993), while a broader range of products have been proven effective at controlling common bunt (Fushtey, 1961). This lack of control of dwarf bunt is likely due to fundamental differences in the infection biology of these species. Common bunt infection occurs beneath the soil surface in less than two weeks at temperatures between 15-22 °C (Fuentes-Dávila, 2002). Thus, a long residual life is not necessary for these products. Dwarf bunt infection, on the other hand, occurs at the soil surface and requires continuous snow cover for at least 60 days (Trione, 1986). Historically, the first fungicides used in the 1940s-1960s for bunt control contained hexachlorobenzene. This compound was banned by the Stockholm Convention in 2004, but has been out of production in the U.S. since 1965 (McGovern, 2004). At the same time, carboxin was registered for common bunt prevention and effectively controlled the disease in the Pacific Northwest, although fungicide resistance was detected (Smiley, 2002).

Current treatments for common bunt and dwarf bunt include demethylation-inhibiting (DMI) triazole fungicides such as difenoconazole belonging to FRAC code 3, which work by inhibiting the biosynthesis of ergosterol, a vital component of the plasma membrane (Smiley, 2002).

Dwarf bunt is not known to persist in Kansas and Oklahoma due to specialized environmental conditions (6 weeks of snow cover) that are rarely met in these states (Peterson et al., 2009, Goates et al., 2011). Current recommendations for common bunt management in

Kansas include the use of certified seed, applications of seed treatment fungicides, and avoidance of late planting (when soil temperatures are low and favorable for bunt infection). The current diagnostic standard for differentiating the pathogens that cause common bunt from *T. controversa* (causal agent of dwarf bunt) is based on differences in spore ornamentation, specifically the depth of reticulations on the surface of *T. tritici* and *T. controversa*. *T. controversa* is believed to have deeper reticulations. The recent detection of spores in Kansas that are morphologically similar to *T. controversa* raises several questions, including whether our current management recommendations can still be utilized for these isolates. The objectives of the present study were to 1.) determine if products containing difenoconazole are necessary to control these novel isolates of *T. tritici* and 2.) to compare the efficacy of currently labeled products in Kansas for the control of novel isolates and *T. laevis*.

Materials and Methods

Field plot establishment

This study was conducted across three environments in Kansas between 2021-2023. Planting was delayed until soil temperatures reached an optimal point for infection (below 45 ^{o}F). Soil temperatures were monitored through the K-State Research and Extension weather station network (Kansas Mesonet). Each location had a Kansas Mesonet weather station within 25 km of the fields where plots were established.

In 2022, a single location was established at the Rocky Ford Experiment Field Station in Manhattan, KS on January 14th, 2022. Established plots were 5' x 25' ft and were planted with a 5-row cone planter at a seeding rate of 1 million seeds per acre. The experimental design in 2022 was a factorial arranged randomized complete block design with four replications per treatment.

The hard red winter wheat cultivar ‘SY Wolf’ was selected for this study based on previous data indicating susceptibility to common bunt. Plots were harvested on July 18th, 2022 using a Wintersteiger Research plot combine.

During the 2022-2023 wheat season, the experiment was established in both Manhattan, KS at the Kansas State University Rocky Ford Experiment Station and the Kanas State University North Central Experiment Field in Belleville, KS using a factorial design in a split-plot arrangement with inoculum as the whole plot and seed treatment as the sub-plot to avoid cross-contamination between isolate treatments. Four replications were established per treatment. In the 2022-23 experiment at the Rocky Ford location, wheat was planted on December 7th, 2022. A Great Plains 606NT 6-row no-till drill modified by Kincaid for plot planting was used to establish 5' x 25' ft plots with a seeding rate of 1 million seeds per acre. Compressed air was used away from the field to clean out the planter between isolate treatments to minimize cross-contamination of isolates. Plots were harvested using a small plot Kincaid 8XP combine on July 7th, 2023. At the Belleville location, wheat was planted on December 12th, 2022 using the same planter, plot size, and seeding rate of 1 million seeds per acre with the same sanitation methods and experimental design as the Rocky Ford location in 2022-23. Plots were harvested on July 13th, 2023. Fertilizers and herbicide were applied in all experimental locations according to standard agronomic practices common in the region. No foliar fungicides were applied to these experiments.

Seed inoculations

Two isolates of common bunt, isolated from grain grown in Kansas, were chosen based on pilot studies of reticulation depth and germination requirements. One isolate (KS0002) was

morphologically similar to the species *T. laevis* (smooth-walled), and the other was similar to *T. tritici* (KS0017) but with longer-than-expected reticulations. These isolates were used to inoculate untreated seed of the cultivar 'SY Wolf'. Infested seed was achieved by breaking open sori to release teliospores, then combining seed and spores in plastic containers, evenly coating clean seed at a rate of 1% (w/w) as described by Wilcoxson and Saari for field inoculations (Goates, 1996). Non-inoculated seed was used as a control.

Seed treatments

Seed treatments were selected for this study based on previously documented efficacy for controlling dwarf bunt (Dividend Extreme) or due to their availability in the market in Kansas during the years 2020-2023 (CruiserMaxx Vibrance Cereals and Raxil Pro MD). After inoculating, seed was treated with one of three seed treatment products: Cruiser Maxx Vibrance Cereals (Group 3, 4, 7; Active ingredients: Difenoconazole 3.34%, Mefenoxam 0.86%, Thiamethoxam 2.78%) at a rate of 0.074 fl oz/ 450 g of seed, Raxil Pro MD (Group 3, 3, 4; Active ingredients: Prothioconazole 1.47%, Tebuconazole 0.29%, Metalaxyl 0.59%) at a rate of 0.05 oz / 450 g of seed, or Dividend Extreme (Group 3; Active ingredients: Difenoconazole 32.8%) at a rate of 0.02 oz / 450 g of seed. A non-treated control was also included in this study. Products were added to the individual plastic buckets at specified rates along with grain, shaken for even coverage, then treated seed was placed in paper bags to dry overnight before packaging.

Field Evaluations

In each environment, plant height was evaluated at flowering (Feekes 10.5) by averaging three height (cm) measurements (randomly taken from the front, middle, and back of plots).

Measurements were taken from the soil to the tip of the awns. The number of bunted heads out of fifty plants per plot was evaluated at Feekes 11.4 by indiscriminately selecting 50 heads per plot and opening the glumes to check for sori. Plant height at the Rocky Ford location during the 2021-2022 season was measured on June 2nd, 2022. The number of bunted heads per plot was evaluated on July 5, 2022 at Feekes 11.4. The average plant height per plot at the Rocky Ford location during the 2022-2023 season was determined May 26th, 2023 at Feekes 11.1. The number of smutted heads per plot was evaluated once on June 16th and again June 30th to evaluate the disease progression as the wheat matured. The average plant height per plot at the Belleville location during the 2022-2023 season was determined using the method previously described. The number of bunted heads per plot was evaluated on June 20th and June 29th, 2023.

Data Analysis

Yield, height, and incidence data were analyzed by location:year using a mixed model analysis of variance using the GLIMMIX procedure in SAS v. 9.4. Mean separation of significant treatment effects was carried out with Fisher's least significant difference (LSD) utilizing the *lsmeans* statement (alpha = 0.05). Bunt incidence data was analyzed with the Poisson distribution while yield and height data were analyzed assuming a Gaussian distribution. A small numeric constant was added to the incidence data to deal with the large number of zeros in this response variable. Inoculum treatment, seed treatment and the interaction of those effects were considered fixed effects while block was considered a random effect. Years and locations were analyzed independently due to the change to a split-plot arrangement in the 2022-23 season.

Results

Environmental conditions at planting

Soil temperature has been described as an important variable for bunt infection. For each location, planting was delayed until soil temperatures were below 45 ºF (7.2 ºC). Figure 3-1 represents average, minimum, and maximum daily 5 cm soil temperatures for the week prior to and week directly following planting date. In Manhattan during the 2021-22 season the average soil temperature on the day of planting was 0.3 ºC (min = 0 ºC, max = 0.5 ºC). In the week following planting the average 5 cm soil temperatures reached a maximum of 1.4 ºC. In Belleville and Manhattan during the 2022-23 season average soil temperatures at planting were 4.3 ºC (min = 3.3 ºC, max 6.1 ºC) and 4.3 ºC (min = 3.3 ºC, max 5.3 ºC), respectively. In the week following planting the maximum soil temperature reached 6.7 ºC.

Field trail results in the 2021-2022 growing season

During the 2021-2022 growing season at the Manhattan, KS location, all three seed treatments effectively reduced the incidence (INC) of common bunt nearly 100% when compared to the non-treated control for each of the two isolates included in this study (Figure 3-2). Mean INC in the untreated plots inoculated with *T. tritici* (KS0017) was 16% and INC was 9.5% for the untreated plots inoculated with *T. laevis* (KS0002). The effect of inoculum (INOC) and the interaction of INOC and seed treatment (SEEDTRT) were not significant in 2021-22 (Table 3). The main effect SEEDTRT on INC was significant in this year (Table 3), with all treatments significantly reducing INC compared to the non-treated controls (Figure 3-2). There was no significant effect of SEEDTRT or INOC on either yield (bu/A) or height (cm) in this year (Figure 3-2,Table 3). Yields ranged from 22.7 to 14.1 bu/A in 2021-22 while average plant heights ranged from 74.2-78.4 cm. Incidence of bunt (3%) in non-treated plots during this year

was likely due to cross contamination in the planter thus the protocol was modified in the 2022-23 season to decrease the likelihood of cross contamination.

Field trial results in the 2022-2023 growing season

In Belleville, KS during the 2022-2023 season, all three seed treatments effectively controlled INC for each of the two isolates with no bunted heads observed in the treated plots. Means for INC, grain yield (bu/A), and plant height (cm) are summarized in Figure 3-3. Untreated plots inoculated with *T. tritici* (KS0017) and *T. laevis,* (KS0002) had an average incidence of 35% and 18%, respectively. Incidence in the untreated, non-inoculated plots was 0.67%. The effect of seed treatment was significant ($P = 0.011$) while the effect of INOC and the interaction of INOC and SEED treatment did not significantly influence INC. There was no significant effect of SEEDTRT or INOC on either yield (bu/A) or height (cm) in this year (Table 3, Figure 3-3). Grain yields in this location ranged from 14.3-22.9 bu/A while average heights ranged from 47.1-52.5 cm.

Means for INC, grain yield (bu/A), and plant height (cm) are summarized for the Manhattan, KS 2023 location in Figure 3-4. Similar to the other two locations that were previously described, both seed treatments resulted in complete control of each of the isolates. The effect of SEEDTRT was significant (Table 3) while the effect of INOC and the interaction of INOC and SEEDTRT were not significant. An INC of 11.0%, 21.0%, and 2.0% were observed in the untreated plots for the non-inoculated, *T. laevis,* and *T. tritici* inoculate plots, respectively. In this location, there was a significant effect of SEEDTRT on grain yield ($P = .002$) with the untreated plots inoculated with *T. laevis, T. tritici* or non-inoculated resulting in the lowest grain yields, 86.1, 86.8, and 84.5, respectively. This location had the highest yields in

the study ranging from 84.5 to 90.0 bu/A. There was no significant difference in plant heights for any of the treatments (Figure 3-4).

Discussion

Based on results from this study conducted across three environments in Kansas between 2021-2023, both *T. laevis* and the abnormal isolate of *T. tritici*, with deeper-than-expected reticulations, can be effectively controlled with currently marketed seed treatment fungicides: Raxil Pro MD, CruiserMaxx Vibrance Cereals, and Dividend Extreme. Each of these products was highly effective at reducing disease levels and none provided improved control over the others. Given the phenotypic difference between known isolates and these new abnormal isolates, this is a critical finding to support management of this disease in Kansas. Furthermore, this study confirms the efficacy of three commonly available seed treatments, CruiserMaxx Vibrance Cereals, Raxil Pro MD, and Dividend Extreme for both the management of *T. laevis* and *T. tritici* in Kansas.

Previous work demonstrated the efficacy of Raxil Pro MD (prothioconazole 1.47% + tebuconazole 0.29% + metalaxyl 0.59%) for the management of *T. laevis* (Johnston, 2005b, Johnston, 1999b, Johnston, 2001b), although less information is available about the efficacy of the other two products. Of the three products tested, Dividend Extreme (difenoconazole 7.73% + mefonoxam 1.93%) and CruiserMaxx Vibrance Cereals (sedaxane .72% + difenoconazole 3.34% + mefenoxam 0.86% + thiamethoxam 2.78%) are labeled for *T. controversa* control, while Raxil Pro MD is only labeled for common bunt (stinking smut). The label of the former products for dwarf bunt is likely due to the inclusion of the active ingredient difenoconazole. These products differ in the concentration of this active ingredient, with Dividend Extreme containing 0.77 lbs of

difenoconazole per 2.5 gallons and CruiserMaxx Vibrance Cereals containing 0.31 lbs of difenoconazole in the same volume. The product Dividend (difenoconazole 32.8%), which has proven effective for dwarf bunt control (Sitton, 1993) has a much higher amount of the active ingredient difenoconazole (3.1 lbs per gallon). Dividend was released in 1992 and does not appear to be widely marketed in 2023. Although they appear to work well for common bunt in the experiment presented here, an open question exists as to whether these newer formulations effectively manage the pathogen that causes dwarf bunt.

Low-level incidence detected during the 2021-2022 growing season at Manhattan in the Dividend Extreme plots, is likely due to unintentional cross-contamination of seed within the planter. Previous tests using this seed treatment (Sitton, 1993), as well as the results of the 2022-2023 environments, support this hypothesis. Sanitation precautions were improved during the 2022-2023 growing season to eliminate or significantly reduce further occurrences. Nevertheless, in all seasons, contamination was observed in the non-inoculated plots. In 2021, the experiment was organized as a randomized complete block design with all treatments represented in each block. As seed was inoculated with teliospores prior to planting, there was likely teliospore contamination of the planter between plots which infested un-inoculated seed. In the second year, the trial was re-organized to allow for compressed air to be blown through the planter with the goal of removing teliospore cross contamination. Although it appeared to help, there was still significant disease in the uninoculated treatments. Future methods for planter cleanout should be evaluated.

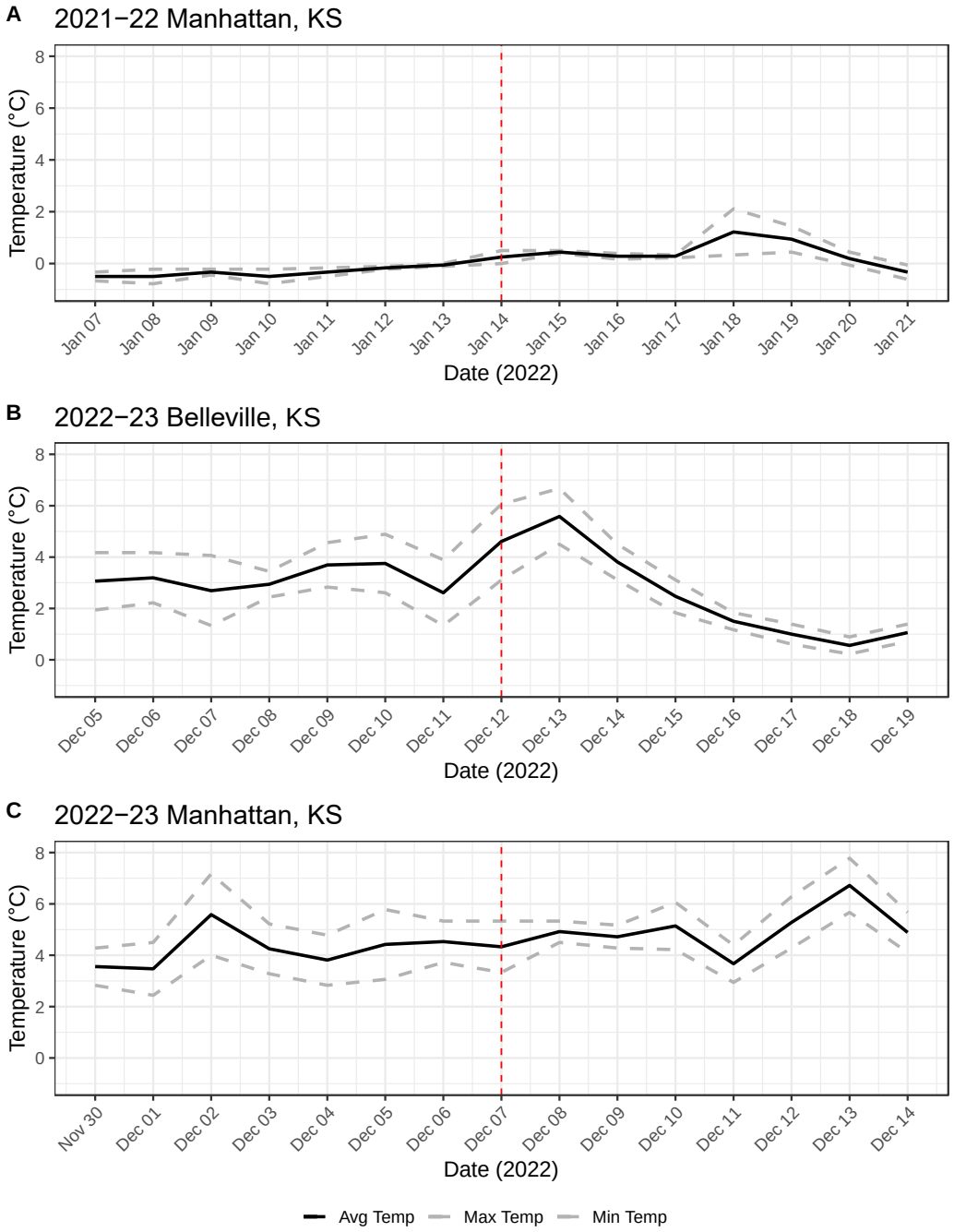
These experiments were planted after the optimum planting date for winter wheat in the region in each of the three locations with a goal of optimizing infections. Based on the literature, the goal of this study was to plant the trial after soil temperatures were below 45 °F (Goates,

1996, Swinburne, 1963). Late planting can generally result in a yield penalty for winter wheat through reduced fall tillering and decreased kernel weight (Dahlke, 1993). In Belleville during the 2022-23 season, the crop was impacted by drought with emergence delayed until late February to early March. Bunt infection still occurred, however, indicating that there was sufficient soil moisture for teliospore germination and infection. Future work should evaluate the current management recommendations that are based on soil temperature and soil moisture.

Table 3 Probability values from generalized linear mixed model analysis of main effects and interactions of the main effects on incidence, yield (bu/A), and plant height (cm).

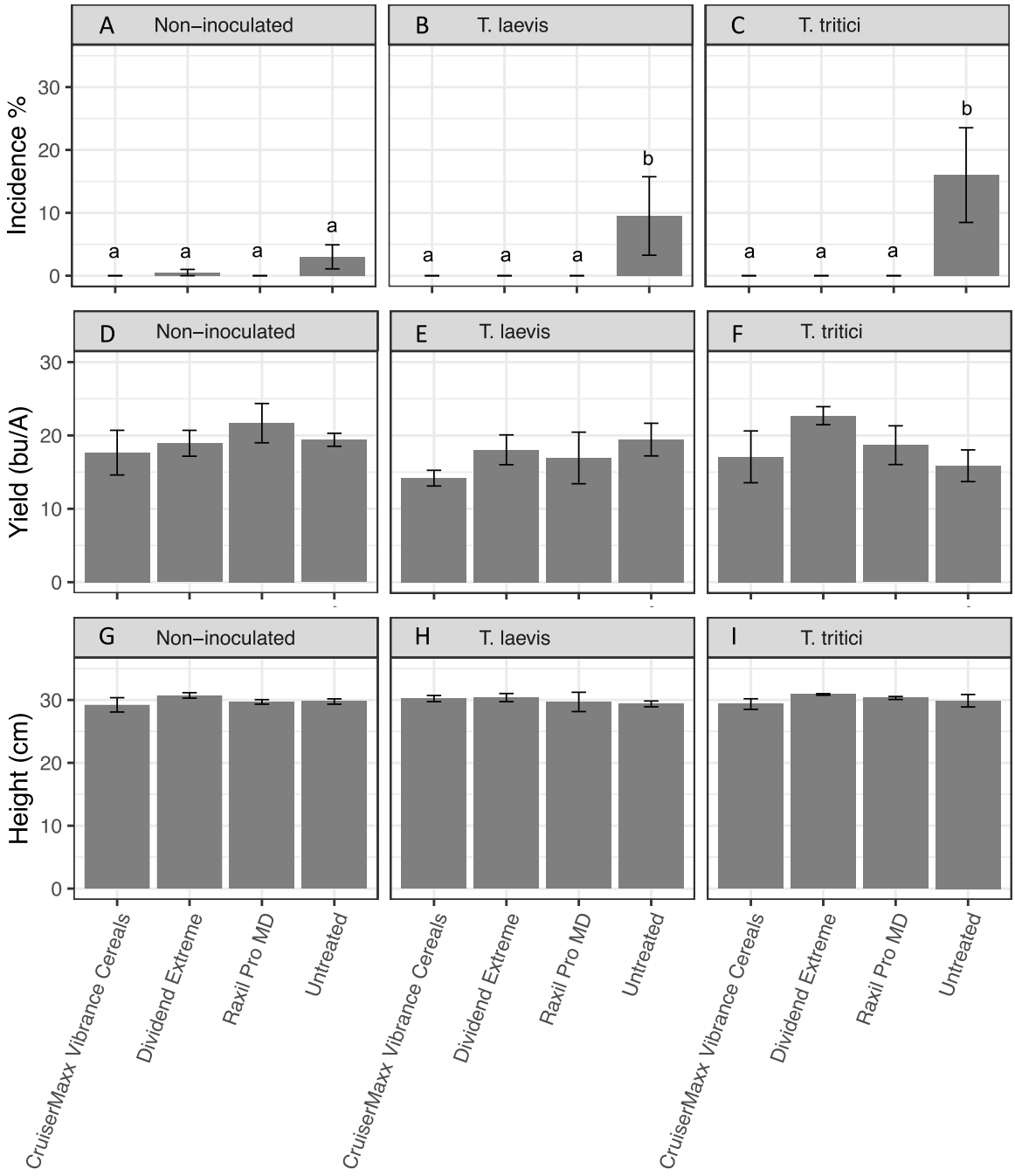
Location	Year	Factor	Incidence	Yield (bu/A)	Height (cm)
Manhattan	**2021-22**	SEEDTRT	<0.001	0.286	0.239
		INOC	0.320	0.387	0.863
		SEEDTRT*INOC	0.281	0.520	0.911
Manhattan	**2022-23**	SEEDTRT	0.003	0.165	0.371
		INOC	0.120	0.747	0.530
		SEEDTRT*INOC	0.020	0.976	0.417
Belleville	**2022-23**	SEEDTRT	0.011	0.002	0.102
		INOC	0.973	0.618	0.170
		SEEDTRT*INOC	0.989	0.1820	0.542

Figure 3-1 Soil temperature at and around planting in each season of field experiments.



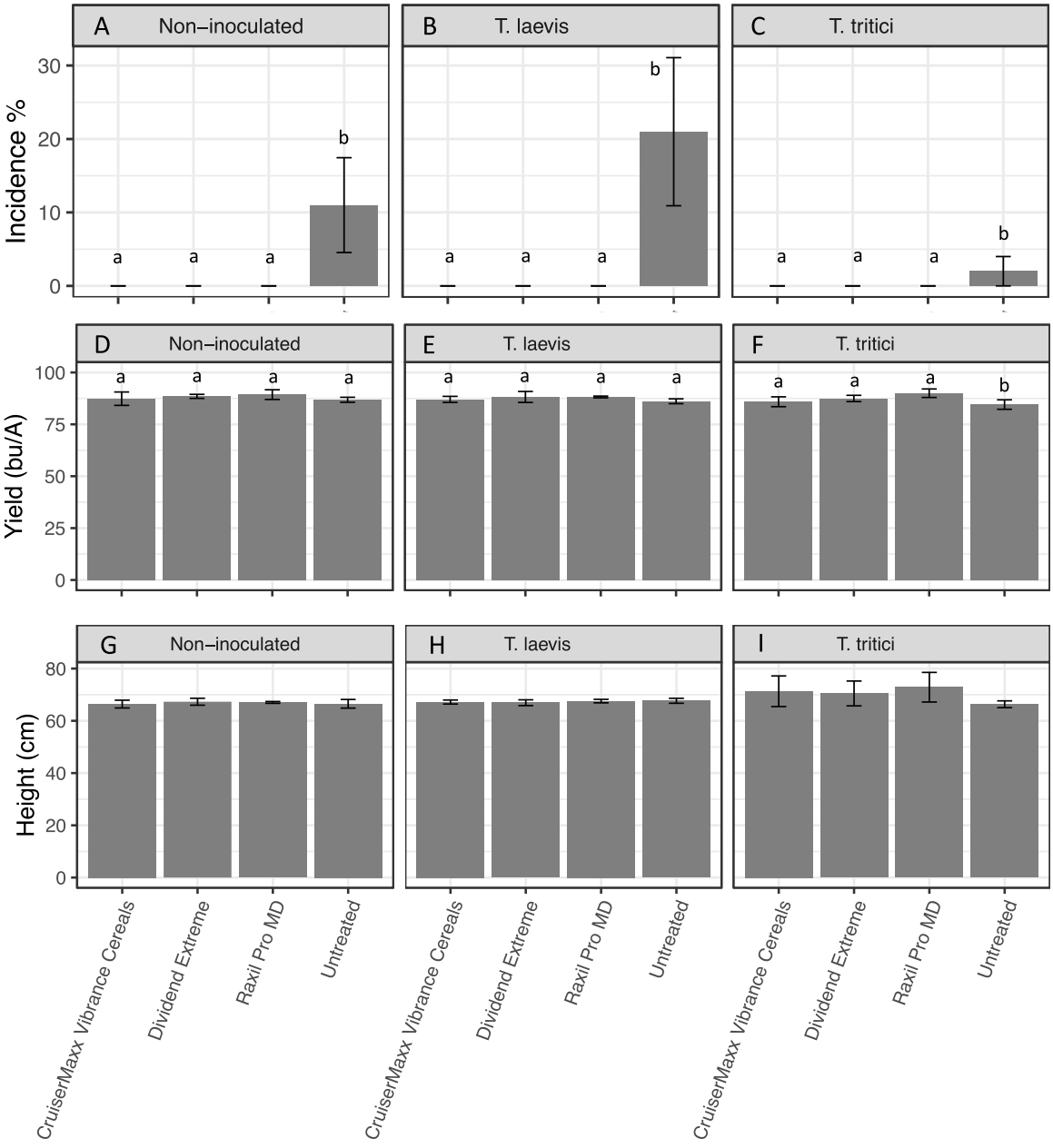
Daily average soil temperatures at 5 cm soil depths during the planting period for each season that these experiments were conducted. Average soil temperature is represented by the thick black lines while daily minimum and maximum temperatures are represented by broken gray lines. Vertical red lines indicate the date of planting for each location: year, respectively.

Figure 3-2 Results from field experiment in Manhattan, KS 2021-22 season.



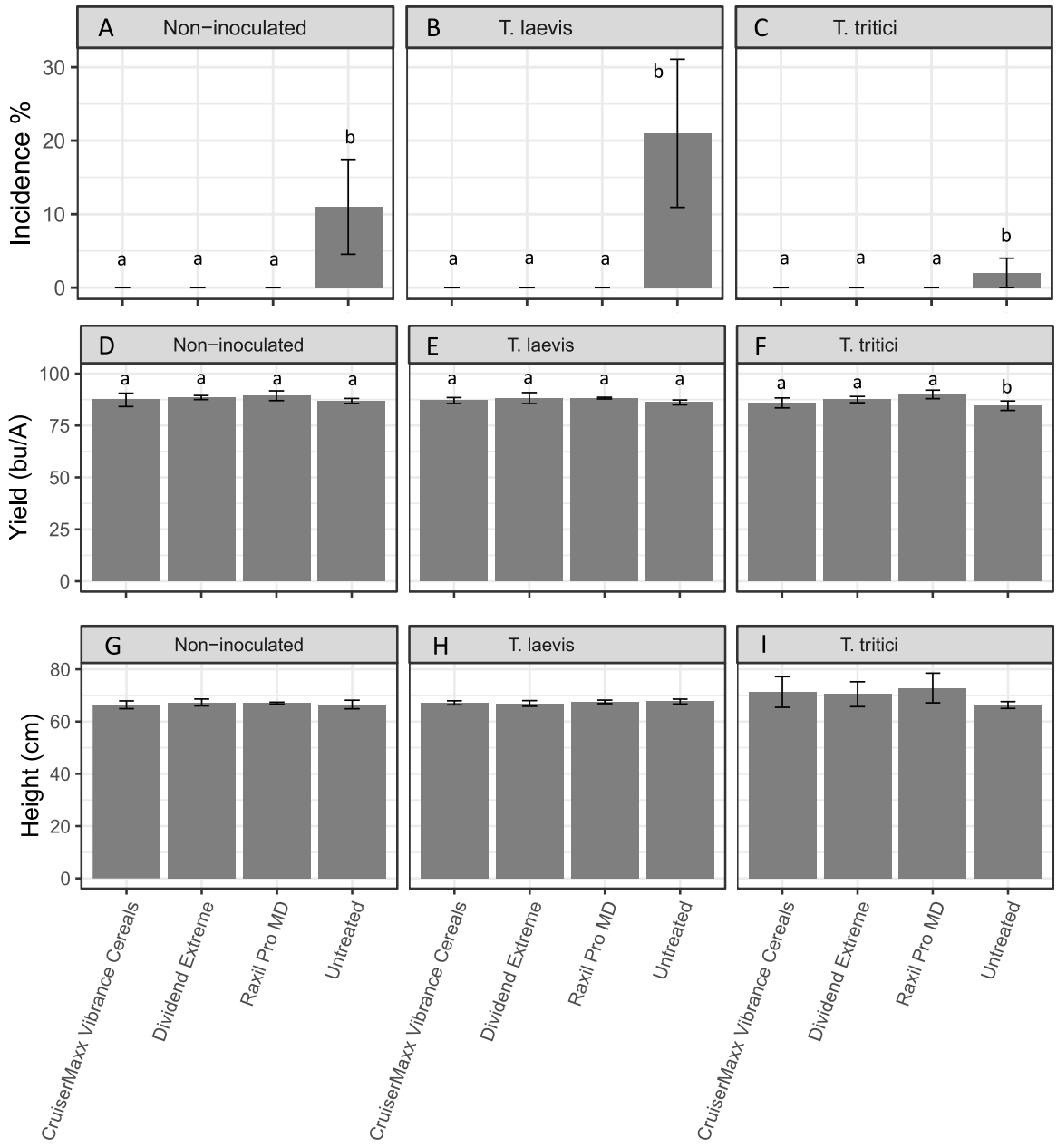
Mean incidence (%), yield (bu/A) and plot height (cm) for each of the combinations of isolate treatment (*T. laevis* (KS0002), *T. tritici* (KS0017) or non-inoculated) and each of four fungicide treatments (CruiserMaxx Vibrance Cereals, Dividend Extreme, Raxil Pro MD, and Untreated) in Manhattan in 2022. Means with different letters are statistically different according to Fishers least significant difference (LSD). Plots with no letters represent main effects that were not significant.

Figure 3-3 Results from field experiment in Belleville, KS in 2022-23 season.



Mean incidence (%), yield (bu/A) and plot height (cm) for each of the combinations of isolate treatment (*T. laevis* (KS0002), *T. tritici* (KS0017) or non-inoculated) and each of four fungicide treatments (CruiserMaxx Vibrance Cereals, Dividend Extreme, Raxil Pro MD, and Untreated) in Belleville in 2023. Means with different letters are statistically different according to Fishers least significant difference (LSD). Plots with no letters represent main effects that were not significant.

Figure 3-4 Results from field experiment in Manhattan, KS 2022-23 season.



Mean incidence (%), yield (bu/A) and plot height (cm) for each of the combinations of isolate treatment (T. laevis (KS0002), T. tritici (KS0017) or non-inoculated) and each of four fungicide

treatments (CruiserMaxx Vibrance Cereals, Dividend Extreme, Raxil Pro MD, and Untreated) in Manhattan in 2023. Means with different letters are statistically different according to Fishers least significant difference (LSD). Plots with no letters represent main effects that were not significant.

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Appendix A - Supplementary Material

Table A- 1 List of *Tilletia* spp. isolates referenced in this thesis.

Current ID	Species	Suggested ID	Location (or notes if location unknown)	Year	Experiment
KS0001	Unknown	*T. laevis*	Kansas	2020	Germ, Ret, Diam
KS0002	Unknown	*T. laevis*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
KS0004	Unknown	*T. laevis*	Kansas	Unknown	Germ, Ret, Diam
KS0006	Unknown	*T. laevis*	Kansas	2012	Germ, Ret, Diam
KS0007	Unknown	*T. laevis*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
KS0008	Unknown	*T. laevis*	Kansas	2010	Germ, Ret, Diam
KS0009	Unknown	*T. laevis*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
KS0010	Unknown	*T. laevis*	Kansas	2016	Germ, Ret, Diam
KS0011	Unknown	*T. laevis*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
KS0012	Unknown	*T. laevis*	Kansas (Field)	2021	Germ, Ret, Diam
KS0013	Unknown	*T. laevis*	Kansas	2018	Germ, Ret, Diam
KS0014	Unknown	*T. laevis*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
KS0015	Unknown	*T. laevis*	Kansas (Field)	2021	Germ, Ret, Diam
KS0016	Unknown	*T. tritici*	Kansas	2020	Germ, Ret, Diam
KS0018	Unknown	*T. tritici*	Kansas (Field)	2021	Germ, Ret, Diam
KS0019	Unknown	*T. tritici*	Kansas	2018	Germ, Ret, Diam
KS0020	Unknown	*T. tritici*	Kansas (Field)	2021	Germ, Ret, Diam
KS0021	Unknown	*T. laevis*	Kansas	2017	Germ, Ret, Diam
OK0023	Unknown	*T. tritici*	Harper Co. OK	2020	Ret, Diam
KS0024	Unknown	*T. tritici*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
KS0024	Unknown	*T. tritici*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
KS0025	Unknown	*T. tritici*	Kansas (Field)	2021	Germ, Ret, Diam
KS0025	Unknown	*T. tritici*	Kansas (Field)	2021	Germ, Ret, Diam
KS0027	Unknown	*T. tritici*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
KS0027	Unknown	*T. tritici*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
OK0029	Unknown	*T. tritici*	Alva 72	2020	Ret, Diam
OK0029	Unknown	*T. tritici*	Alva 72	2020	Ret, Diam
OK0030	Unknown	*T. tritici*	Guymon 60	2020	Ret, Diam
OK0030	Unknown	*T. tritici*	Guymon 60	2020	Ret, Diam
KS0031	Unknown	*T. laevis*	Great Bend 146	2020	Ret, Diam
KS0031	Unknown	*T. laevis*	Great Bend 146	2020	Ret, Diam
KS0032	Unknown	*T. tritici*	Great Bend 162	2020	Ret, Diam
KS0032	Unknown	*T. tritici*	Great Bend 162	2020	Ret, Diam
KS0033	Unknown	*T. tritici*	Wichita 133	2020	Ret, Diam
KS0033	Unknown	*T. tritici*	Wichita 133	2020	Ret, Diam
KS0034	Unknown	*T. tritici*	Wichita 105	2020	Ret, Diam
KS0034	Unknown	*T. tritici*	Wichita 105	2020	Ret, Diam
KS0035	Unknown	*T. tritici*	Wichita 99	2020	Ret, Diam
KS0035	Unknown	*T. tritici*	Wichita 99	2020	Ret, Diam

UU0038	TCK (22)	*T. controversa*	Blair Goates	Unknown	Ret, Diam
UU0038	TCK (22)	*T. controversa*	Blair Goates	Unknown	Ret, Diam
KS0039	Unknown	*T. controversa*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
KS0039	Unknown	*T. controversa*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
KS0040	Unknown	*T. tritici*	Kansas	2020	Ret, Diam
KS0040	Unknown	*T. tritici*	Kansas	2020	Ret, Diam
UU0041	Unknown	*T. laevis*	Unknown	Unknown	Germ, Ret, Diam
UU0041	Unknown	*T. laevis*	Unknown	Unknown	Germ, Ret, Diam
UU0042	Unknown	*T. laevis*	Unknown	Unknown	Germ, Ret, Diam
UU0042	Unknown	*T. laevis*	Unknown	Unknown	Germ, Ret, Diam
KS0043	Unknown	*T. laevis*	Kansas	2021	Germ, Ret, Diam
KS0043	Unknown	*T. laevis*	Kansas (Greenhouse)	2021	Germ, Ret, Diam
MI0076	Unknown	*T. controversa*	Michigan	2020	Germ, Ret, Diam
MI0076	Unknown	*T. controversa*	Michigan	2020	Germ, Ret, Diam
MI0078	Unknown	*T. controversa*	Michigan	2020	Germ, Ret, Diam
MI0078	Unknown	*T. controversa*	Michigan	2020	Germ, Ret, Diam
MI0080	Unknown	*T. controversa*	Michigan	2020	Germ, Ret, Diam
MI0080	Unknown	*T. controversa*	Michigan	2020	Germ, Ret, Diam
UU0087	Unknown	*T. laevis*	Unknown	Unknown	Germ, Ret, Diam
UU0087	Unknown	*T. laevis*	Unknown	Unknown	Germ, Ret, Diam
UU0088	Unknown	*T. laevis*	Unknown	Unknown	Germ, Ret, Diam
UU0088	Unknown	*T. laevis*	Unknown	Unknown	Germ, Ret, Diam
UU0089	Unknown	*T. laevis*	Unknown	Unknown	Germ, Ret, Diam
UU0089	Unknown	*T. laevis*	Unknown	Unknown	Germ, Ret, Diam
UT0168	Unknown	*T. controversa*	Logan, UT	2019	Germ, Ret, Diam
UT0168	Unknown	*T. controversa*	Logan, UT	2019	Germ, Ret, Diam
OR0169	Unknown	*T. tritici*	Hermiston, OR	2021	Germ, Ret, Diam
OR0169	Unknown	*T. tritici*	Hermiston, OR	2021	Germ, Ret, Diam
KS0171	Unknown	*T. laevis*	Kansas	2022	Germ, Ret, Diam
KS0171	Unknown	*T. laevis*	Kansas	2022	Germ, Ret, Diam
UT0175	TCK (D1)	*T. controversa*	Nephi, Utah (Goates)	Unknown	Germ, Ret, Diam
WA0176	TCK (D2)	*T. controversa*	Pullman, Washington (Goates)	Unknown	Germ, Ret, Diam
MT0177	TCK (D3)	*T. controversa*	Kalispell, Montana (Goates)	Unknown	Germ, Ret, Diam
ID0178	TCK (D4)	*T. controversa*	Genesee, Idaho (Goates)	Unknown	Germ, Ret, Diam
WA0179	TCK (D5)	*T. controversa*	Mt. Hope, Washington (Goates)	Unknown	Germ, Ret, Diam
WA0180	TCK (D6)	*T. controversa*	Antone, Washington (Goates)	Unknown	Germ, Ret, Diam
UT0181	TCK (D7)	*T. controversa*	Logan, Utah (Goates)	Unknown	Germ, Ret, Diam
ID0182	TCK (D8)	*T. controversa*	Preston, Idaho (Goates)	Unknown	Germ, Ret, Diam
ID0183	TCK (D9)	*T. controversa*	Tetonia, Idaho (Goates)	Unknown	Germ, Ret, Diam
UT0184	TCK (D10)	*T. controversa*	Blind Spring, Utah (Goates)	Unknown	Germ, Ret, Diam
ID0185	TCK (D11)	*T. controversa*	Hill City, Idaho (Goates)	Unknown	Germ, Ret, Diam
WA0186	TCK (D12)	*T. controversa*	Mt. Hope, Washington (Goates)	Unknown	Germ, Ret, Diam
ID0187	TCK (D13)	*T. controversa*	Paris, Idaho (Goates)	Unknown	Germ, Ret, Diam
UT0188	TCK (D14)	*T. controversa*	Petersboro, Utah (Goates)	Unknown	Germ, Ret, Diam
ID0189	TCK (D15)	*T. controversa*	Preston, Idaho (Goates)	Unknown	Germ, Ret, Diam

ID0190	TCK (D16)	*T. controversa*	Preston, Idaho (Goates)	Unknown	Germ, Ret, Diam
UT0191	TCK (D17)	*T. controversa*	Blind Spring, Utah (Goates)	Unknown	Germ, Ret, Diam
UU0192	TCK (D18)	*T. controversa*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0193	TCK (D19)	*T. controversa*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0194	T. tritici (T1)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0195	T. tritici (T2)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0196	T. tritici (T3)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0197	T. tritici (T4)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0198	T. tritici (T5)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0199	T. tritici (T6)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0200	T. tritici (T7)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0201	T. tritici (T8)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0202	T. tritici (T9)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0203	T. tritici (T10)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0204	T. tritici (T11)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0205	T. tritici (T12)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0206	T. tritici (T13)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0207	T. tritici (T14)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0208	T. tritici (T15)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0209	T. tritici (T16)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0210	T. tritici (T17)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0211	T. tritici (T18)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0212	T. tritici (T19)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0213	T. tritici (T20)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0214	T. tritici (T21)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0215	T. tritici (T22)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0216	T. tritici (T23)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0218	T. tritici (T25)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0219	T. tritici (T26)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0220	T. tritici (T27)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0221	T. tritici (T28)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0222	T. tritici (T29)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0223	T. tritici (T30)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0224	T. tritici (T31)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0225	T. tritici (T32)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0226	T. tritici (T33)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0227	T. tritici (T34)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0228	T. tritici (T35)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0229	T. tritici (T36)	*T. tritici*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0230	T. laevis (L1)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0231	T. laevis (L2)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0232	T. laevis (L3)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0233	T. laevis (L4)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0234	T. laevis (L5)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0236	T. laevis (L7)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam

UU0237	T. laevis (L8)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0238	T. laevis (L9)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0239	T. laevis (L10)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0245	T. laevis (L16)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0246	T. laevis (L17)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0247	T. laevis (L18)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0248	T. laevis (L19)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0249	T. laevis (L20)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
UU0250	T. laevis (L21)	*T. laevis*	Culture Collection (Goates)	Unknown	Germ, Ret, Diam
KS00251	Unknown	*T. tritici*	Kansas	2018	Ret, Diam
KS00252	Unknown	*T. tritici*	Kansas	2020	Ret, Diam
UT0253	TCK (D1)	*T. controversa*	Nephi, Utah (Goates)	Unknown	Ret, Diam
ID0254	TCK (D4)	*T. controversa*	Genesee, Idaho (Goates)	Unknown	Ret, Diam
KS0255	Unknown	*T. laevis*	Kansas	2020	Ret, Diam
KS0256	Unknown	*T. laevis*	Kansas	2017	Ret, Diam