Tracking the Development of Common Bunt Colonization Throughout the Plant Life Cycle in Compatible and Incompatible Reactions Using qPCR

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Abstract

Common bunt, caused by *Tilletia caries* (A.P. de Candolle) L.-R. & C. Tulasne (syn. *T. tritici* (Bjerk.) G. Winter), and *T. laevis* J.G. Kühn (syn. *T. foetida* (Wallr.) Liro), is a reemerging disease that mainly impacts wheat. A seedborne pathogen, it germinates under the soil, infects the new coleoptiles, and colonizes the plant asymptomatically until heading at which point it replaces healthy ovarian tissue with balls of black spores that mature into lookalike kernels called sori or bunt balls. The sori break open during harvest, contaminating seed lots to complete the cycle. Once contaminated, seed will fetch a reduced price or be unsellable. The common bunt fungi are endemic in all wheat-growing regions globally, but they are very closely related to the dwarf bunt fungus, *T. controversa* Kühn which is a quarantine pathogen in some major markets. Due to the morphological similarities, compounded by hybridization between the three fungi, common bunt contaminated grain may be confused for dwarf bunt contaminated grain and be rejected in international markets.

Although an extensive series of seed-treatment fungicides have been released for common bunt control, the most longstanding and useful tool to protect organic systems and volunteer plants and reduce soil banks is the development of resistant varieties. Currently, sixteen major resistance genes named *Bt1-Bt15* and *Btp* have been identified, of which *Bt8* is the most durable gene in the Pacific Northwest. Breeding resistance into new varieties, and breeding resistant varieties for better agronomic performance, may require years as the disease is currently only rated at heading to maturity, one of the final growth stages in a months-long host life cycle. New technology to accurately identify infection is necessary to shorten breeding timelines, in order to continue to combat a pathogen that readily mutates and hybridizes. Developing that technology requires a better understanding of resistance. Thus far, only four studies on the histopathology of incompatible reactions have been published. Only one of them accounted for the specific genotypes of the hosts and pathogens used. The earlier studies agreed with each other that both incompatible and compatible reactions involve colonization of the host to the same degree until 15-21 days post-inoculation, mostly due to new infection events that are swiftly excluded, while the later study described

damage as late as the tillering stage (Zadoks 21). The limited number of studies and the general lack of genotype reporting provides a limited understanding of how the common bunt fungi interact with the host resistance response, which if improved could accelerate the development of new technologies for identification and exclusion to reduce breeding timelines.

To study the difference between incompatible and compatible reactions, one fully susceptible host and one *Bt8* differential were challenged with one of two common bunt races, resulting in 3 compatible reactions and 1 incompatible reaction. Plants were sampled at 4 growth stages, with individual tissues excised and halved and their DNA extracted from one half and the other cleared for microscopy. The objectives of this study were to (i) modify existing molecular approaches to develop a qPCR assay capable of quantifying the amount of common bunt DNA relative to the amount of host wheat DNA in a sample to (ii) determine the extent of earlier infection in two different host genotypes challenged with two different common bunt and (v) corroborate this information with initial microscopy studies to (iv) determine the differences in colonization and defense induction between compatible and incompatible reactions involving two different host and pathogen genotypes each at each studied growth stage races. The final objective was to (v) investigate whether non-destructive samples taken earlier in the plant life cycle could be used as a tool for resistance breeding, to shorten the breeding cycle in the absence of QTL.

The results indicated that there were no statistically significant differences in colonization patterns until the second node stage, at which point colonization was halted in a race-specific manner. The *T.* caries race was unable to cause disease in the incompatible reaction and was only marginally successful in the compatible reaction. Due to a lack of consistent progressions of fungal colonization between compatible reactions of different host genotypes, and a markedly different resistance timeline development between *Bt8* and *Bt10*, this study concludes that not only is resistance race-specific, it is also host-genotype dependent as is compatibility. This necessitates a higher level of genotype reporting for future studies on the subject, and more research into the general timing of resistance in incompatible reactions if a qPCR assay is to be utilized.

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Dedication

This thesis is dedicated to my parents; to my mother who fielded every distress call, every celebratory video call, and every Sunday call. She asked all the right questions, encouraged me in my work, and helped me learn who I was and could be as an academic. Her constant reassurance helped me believe this all was possible. I'm dedicating this, as well, to my father whose pride in me has been such a comfort. I've never had to earn it, and in freely giving me his love and faith he has helped me develop any ambition and confidence I have.

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Introduction

Common bunt or stinking smut, caused by *Tilletia caries* (A.P. de Candolle) L.-R. & C. Tulasne (syn. *T. tritici* (Bjerk.) G. Winter), and *T. laevis* J.G. Kühn (syn. *T. foetida* (Wallr.) Liro) has been one of the most globally significant diseases of wheat (*Triticum aestivum* L.) in recorded history. The fungi colonize the plant with minimal visible symptoms until heading, and are characterized by a "fishy" odor caused by the chemical trimethylamine (Matanguihan et al. 2011; Wegulo 2009; Murray et al. 2009; McNeil et al. 2004; Goates 1996).

The economic implications of common bunt in a wheat crop are considerable, as most granaries will not accept contaminated wheat or will reduce the price paid to the producer (Murray et al. 2009; Laroche et al. 2000; Goates 1996). Levels as low as 0.01-0.05% bunt balls in a wheat lot result in detectable yield and grain quality losses (Menzies et al. 2006; Laroche et al. 2000). Only 5-14 bunt balls per 250 grams of seed result in a "smutty" grade and price docking (Matanguihan and Jones 2011). Quarantines against dwarf bunt complicate the economic impact of common bunt. The similarity of reticulated common bunt and dwarf bunt spores as well as interspecific hybridization between the two can result in false identification of dwarf bunt and rejection of the grain at the elevator (Murray et al. 2009).

There are effective methods for controlling and limiting common bunt infections, primarily the use of fungicides (of which there are 38+ commercial products available which provide nearly 100% control) and seed cleaning (Blöch 2021; Borgen 2020; Wegulo 2009; McNeil et al. 2004; Paveley et al. 1996; Goates 1996). Globally, however, it remains a challenge to fully exclude common bunt in crop production (Jevtić et al. 2021; Madenova et al. 2021; Gaudet and Menzies 2012). While the incidence of common bunt has decreased with increased availability of seed treatments, it remains an imperfect control. These treatments are not universally accessible (Jevtić et al. 2021; Madenova et al. 2021; Carris 2010; Zouhar et al. 2010; Cockerell 1997; Mamluk and

Nachit 1994; Hoffman 1982), especially in situations where fungicides are discontinued. They may have adverse effects on the environment or human health (Goates 1996; Cockerell 1997; Hoffman 1982) and/or are ineffective when improperly used (Mamluk 1997; Goates 1996; Ballinger and Gould 1988). Seed treatments cannot prevent the buildup of inoculum in the soil produced on susceptible volunteer which provides a latent threat (Mamluk 1997; Cockerell and Rennie 1996), and increases the potential for rapid evolution of the pathogens. The lack of mixture partners in very effective fungicides such as Dividend® (Syngenta 2018) increases likelihood of resistance development (Hollomon 2015; Bowyer and Denning 2014; Kuiper 1965; Parry and Wood 1959).

In low-input agricultural systems, some producers may be unable to afford seed treatments. If seeds are planted without treatment for more than 2 years, yield losses can rise to 75-90% loss (Madenova et al. (2021). Therefore, resistant varieties are imperative to common bunt management. However, most of the wheat cultivars currently in production are not adequately resistant to common bunt in the absence of seed treatment. According to Madenova et al. (2021), "there are practically no common bunt resistant wheat varieties in production." Some researchers link this lack of resistance to more frequent common bunt outbreaks in North America (Dhariwal et al. 2021). Additionally, some areas do not have access to resistant varieties (Anderson Onofre 2020).

As environmental concerns result in promotion of organic systems that restrict the use of chemical seed treatments, closer attention must be paid to understanding the disease cycle in order to reduce infection. Planting of unprotected seeds in organic systems has resulted in significant bunt outbreaks (Jevtić et al. 2021; Sedaghatjoo et al. 2021a; Borgen 2020; Zouhar et al. 2010). With changes to the organic standards in Europe in the 2010s, producers must rely on organically-produced seed subject to strict contamination standards which are much harder to produce and access (Matanguihan and Jones 2011; Wolfe et al. 2008).

Detection of common bunt in various plant tissues pre-heading by PCR-based methods has been reported extensively (Zouhar et al. 2010; Roberts et al. 2007; Kellerer et al. 2012; Kochanová et al. 2006, 2004; Eibel et al. 2005; Josefsen and

Christiansen 2002). Other detection methods have also been published, but the industry standard of microscopic identification of spores and culture tests to differentiate common and dwarf bunt remains.

Resistance in wheat to common bunt has classically been thought to be a product of a gene-for-gene interaction, resulting in relatively faster host elongation than fungal colonization, and the inability of the pathogen to infect the growing point. Other resistance mechanisms proposed are the buildup of callose or related metabolites around the infection zone to contain the initial infection (Gaudet et al. 2007), an unknown mechanism halting the development of the fungus in the first true leaf (Woolman 1930), or an unknown mechanism preventing sporulation although the fungus itself is detectable in developing kernels (Griffith et al. 1953). These proposed resistance methods are also occasionally assumed to be evidence of quantitative resistance or innate resistance. While other wheat pathosystems, such as that of *Fusarium* head blight, categorize modes of resistance into types (Ribichich et al. 2000), no such system has been proposed for common or dwarf bunt. This is likely due to insufficient information on the mechanisms of resistance between known genes.

In this review, common bunt is approached from multiple vantages. The taxonomy is explored, as well as the methods of identification and life cycle. The history and current status of disease management is reviewed in depth. The literature on both the infection process and mechanisms of host resistance are thoroughly reported, as are the diagnostic tools that are currently available. Finally, gaps in the literature are determined with the goal of identifying and addressing them.

Taxonomy

T. caries and *T. laevis* are Basidiomycetes in the class Exobasidiomycetes, order Tilletiales, and family Tilletiaceae. They are closely related to other bunts and smuts representing some of the economically most important pathogens in the world, including karnal bunt (*T. indica*) and dwarf bunt (*T. controversa*) (Vánky 2012; Duran and Fischer 1961).The genus *Tilletia* is named after Mathieu Tillet, who used the fungus in 1755 to demonstrate the pathogenicity of parasitic plant diseases (Matanguihan et al. 2011; Goates 1996; Carefoot and Sprott 1967; Fischer and Holton 1957).

As is common in the genus *Tilletia*, there is some confusion in the literature about the taxonomic history resulting in the distinction between common and dwarf bunt. Kühn, Young, Holton, Fischer, Skolko, and Conners all had separate roles in differentiating the two and contributing to the current taxonomy. In 1874, Julius G. Kühn published the name *T. contraversa* (hosted on *Elymus repens*) in reference to dwarf bunt in the same year as the publication of *T. controversa* for the same fungus (the epithet "*controversa*" has become the accepted spelling). Kühn's studies were the first to describe important features that since have been used to distinguish dwarf from common bunt – smaller average spore size with more uniform spherical spore shape, and deeper exospore and delayed germination as compared to common bunt (Bao 2010).

Despite the contributions of Kühn (Kühn 1874), prior to Young's (1935) publication of a distinction and classification of dwarf bunt as a separate race of *T. caries* (published at the time as *T. tritici*), common bunt fungi were still totally undistinguished from the dwarf bunt fungus (Goates 1996; Conners 1954; Young 1935). Holton, as well, categorized dwarf bunt as a race of *T. caries* (Conners 1954; Holton 1941). Dwarf bunt was described in following years on intermediate wheatgrass (and on wheat as an additional host, but not typified) as *T. brevifaciens* by George W. Fischer in 1952, who described a great difference in the spore morphology between the common bunt fungi and "*T. brevifaciens*." In addition to the differences in spore morphology, he considered the difficulty in culturing dwarf bunt, the ineffectiveness of the usual seed treatments for common bunt, and the more soilborne rather than seedborne nature of infection paired with the dwarfing of the infected plants to be sufficient evidence for the separation of the species (Bao 2010; Conners 1954; Fischer 1952). In comparing his "*T. brevifaciens*" samples from several U.S. states and Germany in the 1940s and early 1950 to Young's (1935) original Montanan sample of the purported race of dwarf bunt, Fischer concluded that *T. brevifaciens* was the same species as Young's (1935) collection, with only slight differences in the hyaline sheath. However, it wasn't until 1954 that the causal agent of dwarf bunt was taxonomically clarified (Conners 1954). Conners (1954) studied the relationships between the species of *Tilletia*, and found that *T. brevifaciens* was indistinguishable from Kühn's 1874

publication of *T. contraversa* (now *T. controversa J.G. Kühn [*as *'contraversa'],* in Rabenhorst, Hedwigia 13: 188 (1874)). Due to the established rules of fungal taxonomy, *T. controversa* was and is currently recognized as the correct name for the dwarf bunt fungus.

Ideally, the basis for separation of the common bunt species is spore wall/exospore characteristics; *T. caries* is reticulate while *T. laevis* is smooth (Murray et al. 2009; Fischer and Holton 1957). Complicating the identification and taxonomy, teliospores of the two common bunt fungi are occasionally found within the same sorus (Carris 2010; Saari et al. 1996), and they have been found to hybridize with each other, with dwarf bunt, and with other *Tilletia* species infecting native and nuisance grasses (Carris 2010; Pimentel et al. 2000; Shi et al. 1996; Silbernagel 1964; Holton 1954, 1941), creating a natural gradient of phenotypes between the species (Goates 1996; Holton 1942; Flor 1933). Phylogenetic studies in 1996, 2005, and 2007 failed to fully distinguish the dwarf and common bunt species from each other (Carris et al. 2007; Castlebury et al. 2005; Shi et al. 1996). It was not until a phylogenomic study in 2019 that strong bootstrap support was given to the separation of dwarf and common bunt clades, although this study was published on the basis of few isolates (Nguyen et al. 2019) and as noted by Bao (2010), work in this genus tends to require a large number of isolates to form conclusive results.

There has been confusion surrounding the first valid publications and naming of the two common bunt fungi - one with reticulate spores and one with smooth spores. This confusion has resulted in the use of four names in the literature; *T. caries* and *T. tritici* for the reticulate and *T. laevis* and *T. foetida* for the smooth (Carris 2010). The taxonomy of the common bunt fungi was resolved in 2012 in Kálman Vánky's monograph on the smut fungi. Based on his investigations, the names *T. caries* and *T. laevis* are the most appropriate and most correct with respect to the code of botanical nomenclature. As a result, *T. foetida* and *T. tritici* have been synonymized under the two more correct names (Vánky 2012).

While Vánky's monograph did separate the common bunt fungi from the dwarf bunt fungus, the common bunt and dwarf bunt fungi are occasionally thought to be conspecific. They share almost identical morphologies (Russell and Mills 1994),

genetics (Sedaghatjoo et al. 2021a; Eibel et al. 2005; Russell and Mills 1994, 1993), disease cycles (Carris 2010), physical characteristics (Russell and Mills 1994), serology (Eibel et al. 2005; Banowetz et al. 1984), epidemiologies and control measures (Carris 2010) and indistinguishable internal transcribed spacer (ITS) sequences and similar genome sizes (Sedaghatjoo et al. 2021a; Nguyen et al. 2019; Carris et al. 2007). Those who believe in a conspecific status point out that the two diseases are distinguished by characteristics that are subject to genetic variability within populations - teliospore germination temperatures, teliospore wall morphology, and dwarfing of the wheat plant (Carris 2010; Russell and Mills 1994, 1993). There is a 10% overlap between the morphologies of *T. caries* and *T. controversa*, making identification by one to few spores nearly impossible (Goates 1996). As discussed previously, Russell and Mills' (1994, 1993) work on the karyotypes, germination, spore wall autofluorescence and chromosomal variability supports a conspecific status. So too does work that carefully searched for but could not find species-specific polypeptide patterns (Kawchuk et al. 1988) and work that searched for but could not find unambiguous serological differences (Banowetz et al. 1984). Others have struck a middle ground - reporting that dwarf bunt and common bunt are likely sub-lineages from a common ancestor that are very capable of interspecific hybridization (Shi et al. 1996). However, several studies have (more or less dubiously) claimed to differentiate between dwarf bunt and common bunt on the basis of teliospore characteristics (Trione and Krygier 1977), exospore epifluorescence (Stockwell and Trione 1986), genetics (Liu et al. 2009; Eibel et al. 2005; Kochanová et al. 2004; McNeil et al. 2004), proteomes (Li et al. 2018; Banowetz and Doss 1994), SCAR markers (Gao et al. 2014b), antigens (Gao et al. 2014a) polypeptides (Weber and Schauz 1985), and more recently, genomics (Sedaghatjoo et al. 2021b; Nguyen et al. 2019) and MALDI-TOF MS analysis (Forster et al. 2022). In Xiaodong Bao's 2010 dissertation on the phylogenetics of *Tilletia* species, an important critique of these studies noted that "their conclusions must be assessed in the context of the number and diversity of the isolates that were analyzed." Bao (2010) critiques Liu et al.'s 2009 study for examining only 15 isolates of unreported geographic origin before publishing a declarative method to distinguish common and dwarf bunt by genetic markers.

The taxonomy of dwarf bunt and common bunt extends unofficially to the level of races. It is well established that different races of the fungi react differently with different genotypes of the host, and for this reason the races have been named according to the host genotypes they infect and in the order of their discovery. For example, races of *T. tritici* (discovered when the epithet was still in use) are labeled T-*x*, *T. laevis* are designated L-*x*, and *T. controversa* are designated D-*x* (Goates 1996). The races are identified by their failure or ability to cause disease on a set of single-resistance-gene wheat differentials (Goates 2012).

Common Bunt Biology and Symptoms

T. caries and *T. laevis* are obligate biotrophs; they must parasitize the living host in order to complete their life cycle (Gaudet et al. 2007; Saari et al. 1996; Goates 1996). While common bunt generally infects winter wheat, it may also infect spring wheat (Carris 2010; Murray et al. 2009) and other grass genera such as *Aegilops, Agropyron, Agrostis, Alopecurus, Arrhenatherum, Beckmannia, Bromus, Dactylis, Elymus, Festuca, Holcus, Hordeum, Koeleria, Lolium, Poa, Secale,* and *Trisetum* and × Triticosecale (Goates 1996; Duran and Fischer 1961; Fischer and Holton 1957). Under field conditions, common bunt spores may remain viable for roughly two years (Goates 1996).

The teliospores are spread in the wind under modern agricultural practices such as combine harvesting, which releases the spores where air currents may carry them over long distances (Saari et al. 1996; Fischer and Holton 1957). Common bunt can occur on both winter and spring wheat throughout the wheat growing regions of the world (Goates 1996). As of 1996**,** common bunt was present in Turkey, Syria, Iran, Afghanistan, Iraq, Lebanon, Jordan, Egypt, Nepal, Pakistan, the hill regions of India, Algeria, Morocco, Libya, Ethiopia, South Africa, China, all of Europe, and most countries in South America (except Brazil). Common bunt has not reduced its wide-reaching range in the ensuing 25 years, and has become one of the top bunt diseases in European countries such as Austria (Blöch 2021). However, new infections are most frequently the result of surface contamination of grain. The infection rate for common bunt is usually predictive of the yield loss at a 1:1 ratio, with the percentage of infected tillers

equivalent to the lost yield, as common bunt does not affect tillering (Menzies et al. 2006; Goates 1996).

Spores of common bunt either contain or induce the production of trimethylamine, which imparts a strong "fishy" odor to infected tissue. Trimethylamine, in appropriate concentrations, is combustible and can cause explosions (Murray et al. 2009; Carefoot and Sprott 1967; Fischer and Holton 1957).

The most prominent symptom is the replacement of kernels with sori (also called "bunt balls"), masses of teliospores that are roughly ovoid and resemble wheat kernels. These bunt balls are not readily apparent until after heading, "when sporulation begins in the very young ovary" (Goates 1996). Sori formation may be preceded by the more slight spreading of glumes in infected spikelets rather than uninfected spikelets (Ren et al. 2021b). Additional minor symptoms have been reported. Infected immature spikes may be darker green and longer than healthy spikes, although mature infected spikes will be lighter and slightly more bluish-gray than their healthy counterparts (Murray et al. 2009; Goates 1996; Jones and Clifford 1983). Culms are potentially slightly stunted (though usually not) (Murray et al. 2009; Goates 1996; Jones and Clifford 1983), and the florets may be slightly flared in comparison to healthy wheat (Goates 1996). Older accounts have claimed that the plant is induced to make more bunt balls than it would have produced seed (Fischer and Holton 1957; Barrus 1916). Some have observed infected plants failing to extrude anthers and producing nonviable pollen (Goates 1996). In club wheat, common bunt can cause breaking of the club phenotype and an elongation of the head (Fischer and Holton 1957). Interestingly, although the production of a stunting phenotype is a diagnostic tool for differentiation between dwarf and common bunt, certain races of common bunt on certain varieties of wheat may also cause stunting (Holton and Rodenhiser 1942).

Some secondary effects seem to vary greatly with the variety of wheat and race of the fungus (Thomas 1991; Fischer and Holton 1957). For example, there is disagreement on the importance of the "flecking" symptom. In some plants, a symptom arises in which the leaves are flecked with white spots in the leaf tissue. A lack of flecking in the first leaves has been correlated to disease resistance in Red Bobs

(Kendrick and Purdy 1959), while later scientists reported variance in flecking with host genotype, casting doubt on the reliability of the flecking symptom in characterizing susceptibility. Josefsen and Christiansen (2002) used their nested PCR detection assay to analyze the pathogen content in flecked and non-flecked inoculated tissues - detecting [*T. caries*] (published as *T. tritici*) in both severely flecked leaves and those showing no symptoms. This experiment casts further doubt on the usefulness of this potential symptom.

Symptoms may also be ameliorated or worsened by interactions with the environment (Gaudet et al. 1993; Rodenhiser and Holton 1942). For example, when the same lines were planted in the same manner at four different locations, researchers noticed that their responses to the same inoculum varied with the environment in a statistically significant manner (Gaudet et al. 1993). The same was found in much earlier studies. In planting the same varieties challenged with the same set of specific races of *T. laevis* (referred to at that time as *T. levis*) and *T. caries* (referred to at that time as *T. tritici*) at several locations across the continental U.S., the researchers found that some varieties and some races responded in starkly different manners depending on the usual conditions at different locations and even between years at the same locations (Rodenhiser and Holton 1942). Rodenhiser and Holton (1942) therefore recommended that races of the fungi and resistance of the hosts not be characterized without study in multiple environments and years.

History of Common Bunt Management

Common bunt has attacked wheat crops for at least as long as humans have recorded history. The disease likely originated in the Middle East (the center of origin of wheat) - as evidenced by the frequency of host resistance genes found in the area and the fungal adaptations suitable to environmental conditions in the Middle East (Saari et al. 1996). It is hard to determine how prevalent bunt was in agriculture in pre-recorded history, but the presence of bunt on related grasses indicates that they have always plagued wheat (Fischer and Holton 1957).

Early theories included bunting as a “corruption” caused by harsh sunlight on overly wet grain (Fischer and Holton 1957; Tillet 1755). Carefoot and Sprott (1967)

describe the evolution of early bunt management techniques, performed before the disease was understood to be alive or within human control. Farmers, apparently recognizing the coincidence of the "black dust" between sowing and harvest, began washing their seed with water. Gradually, seed treatments became harsher as substances such as seawater, limewater, and lime slurry were adopted. In the Middle Ages, farmers are said to have used ammoniated horse urine as well as human urine, recorded as believing that substances that could bring a tear to their eye could kill anything. None of these washes were particularly effective.

From the beginning of scientific study, the bunts (along with other cereal smuts) were the most studied as they were "literally robbing the breadbasket of mankind" (Fischer and Holton 1957). Common bunt had severe economic repercussions, severely reducing wheat yields within any large wheat-growing country, but these outbreaks could be highly localized in geography. Therefore, they were devastating to individual regions (Fischer and Holton 1957). Early recordings use "blast, mildew, blight, rust, and brand" to seemingly refer to rusts, smuts, mildews and other diseases interchangeably (Fischer and Holton 1957). Jethro Tull did recognize the "smut" in 1700 - he's quoted as saying "Smuttiness is when grains of wheat, instead of flour, are full of black stinking powder." (Carefoot and Sprott 1967).

The common bunt fungi were originally understood, though not characterized, by Mathieu Tillet, who entered and won a contest to demonstrate the best understanding of the smut of wheat. After analyzing the prevalent theories for the cause of smut at the time, and concluding that none were sufficient explanations, he took to his own experiments. He began by challenging wheat plants to the abiotic conditions thought to cause corruption in a research environment, proving that no bunt developed and thus it could not be abiotic. He also performed trials in which he inoculated seed with the black dust. His experiments demonstrated for the first time that the "black dust" was responsible for the infection - and provided the first management techniques. In washing the seeds with water, cattle urine, lye solutions, lime and salt, and finally, copper sulfate he demonstrated that each of these solutions could reduce (but not control) the incidence of bunt. His name was, then, immortally tied to what he originally understood as the black dust containing the (unproven, but rationally concluded to be

systemic) “virus”. At the time, early in the beginning of germ theory, “virus” was the term for all plant diseases (Matanguihan et al. 2011; Carefoot and Sprott 1967; Fischer and Holton 1957; Tillet 1755).

Prévost demonstrated the fungal nature of the pathogen in 1807, and investigated the control of the stinking smut with copper and copper salts (Russell 2005; Carefoot and Sprott 1967; Fischer and Holton 1957; Prévost 1807). This scientific work, even in conjunction with Tillet’s study, was not enough to convince the larger body of science that the disease was biotic and not caused by a stagnation of sap (Fischer and Holton 1957). Therefore Prévost’s copper seed-treatment suggestions were not implemented until the middle of the nineteenth century (Carefoot and Sprott 1967), only after the Irish potato famine had brought crop disease to the forefront of agricultural science (Russell 2005). In 1815, De Candolle recognized that the black dust was a fungus, which he named *Uredo caries.* This still was unconvincing to the larger scientific community (Fischer and Holton 1957).

The seminal study naming *Tilletia* (after Mathieu Tillet) was performed by the Tulasne brothers, who studied the spore germination of the fungus in detail and, for the first time, correctly understood the primary and secondary sporidia as well as the formation of H-bodies (Carefoot and Sprott 1967; Fischer and Holton 1957). Afterwards, Brefeld published numerous works greatly enhancing the understanding of the smut fungi in general (Fischer and Holton 1957).

Beginning in 1807, Prévost began publishing on the effectiveness of copper sulfate as a seed treatment (Matanguihan et al. 2011). After the widespread adoption of copper treatments in the 1850s-1900s it became an issue that the copper products, when improperly applied, were toxic to the wheat as well. As necessary, the chemistry available advanced and by about 1900 formaldehyde was more widely recommended. Chemists advanced the field further with more effective dusts - often made with mercury or colloidal compounds that were poisonous and had to be treated carefully (Carefoot and Sprott 1967).

Throughout the history of common bunt management, breeding for resistance has been of major importance (Goates 1996; Woolman 1930). In 1930, Woolman, writing in *Phytopathology* from Pullman, WA, reported that resistant varieties had

nearly "solved most satisfactorily" the problem of common bunt. He noted that yield of resistant varieties must be competitive with non-resistant preferred cultivars/varieties in order to be economically feasible.

In Pullman, the search for resistance to common bunt began in 1914 (Gaines 1925). Globally, researchers had begun diligently working on the finding and development of resistant varieties (Tisdale et al. 1925), although the confines of gene-for-gene resistance were not yet known. To better understand the resistance that the breeding program in the Pacific Northwest was screening for, E.F. Gaines and colleagues began studying the genetic basis for bunt resistance (Gaines 1923, 1920). In 1928, he published two papers establishing the modern understanding of race specialization among the common bunt fungi (Gaines 1928b, 1928a). This work was expanded in 1933, when an additional study corroborated previous work and reconsidered the idea of "physiologic forms," suggesting that the term was not entirely accurate (Flor 1933). The works of Gaines and Flor were instrumental in developing the concerted effort to breed away the pathogen. As breeding programs developed new varieties, though, each variety seemed to have a limited lifespan of resistance before a new virulent race of common bunt was able to overcome it (Matanguihan et al. 2011; Shepherd 1980). Although Gaines successfully bred and distributed two high-quality, bunt-resistant varieties in 1915 and 1927, the short respite from smut pressure waned between 1923-1928 (Shepherd 1980). However, as new resistance genes were discovered and deployed, their prevalence shifted the population structures of the bunt races in a given area. The alternating successes of pathogen and host suggested, then, that a gene-for-gene reaction was at play. Since the development of that knowledge, breeders have worked to define races and genes and continuously improve the available resistant germplasm (Matanguihan et al. 2011).

Despite concerted breeding efforts and the development of seed washes, before modern systemic seed treatments were developed, losses from common bunt could be devastating. In Europe, losses upwards of 50% were common and occasionally the crop was irrecoverable (Fischer and Holton 1957). Interestingly, up to 1917, bunt caused losses to producers due to "bunt explosions," found to be the result of friction igniting high concentrations of the flammable spores (Fischer and Holton 1957). In England,

33% of the 1921 wheat harvest were bunt balls (Marshall 1960). In the Pacific Northwest of the USA, before seed treatments in the period between 1951-1955, losses averaged more than 4.5 million dollars (the equivalent of 43 million dollars in 2021), continuing a trend of staggering losses recorded from the early 1900's. At its most severe, a 1921 outbreak in the Dakotas and Montana alone resulted in an 8 million dollar loss (the equivalent of $106 million in 2021). After these years of intense loss, copper carbonate was promoted to control bunt. A breeding program was officially put in place (after 1929), and smut-free seed and a new chemical called 'New Improved Ceresan' were deployed in the 1930s (Fischer and Holton 1957).

In Australia, as with every wheat-producing region of the world, common bunt posed a significant threat in the early 1900s. By 1927, a seed treatment program was in effect, but the seed treatments used provided ineffective control and caused a 25% seed mortality (Fischer and Holton 1957).

The dockage system on contaminated grain was described in 1957 as a penalty system for "smutty wheat" (wheat that smelled of bunt or was contaminated with spore balls or spores in excess of 14 average sized bunt balls in 250 kernels of wheat). Dockage amounts were based on the weight lost when the wheat was scoured to remove the bunt, expressed as a percentage. A 1% dockage meant that 1 in every 100 pounds must be removed by cleaning and a $1.00/ton washing fee posted as well as a freight charge (Fischer and Holton 1957).

Farmers were largely at the mercy of common bunt until the advent of chemical seed treatments in the mid-1900s (Matanguihan et al. 2011). In the 1950s, polychlorinated benzene (polychlorobenzene) fungicides were released to mitigate the effects of both seedborne and soilborne inocula, and organic mercury fungicides were released to eliminate the threat of seedborne infection (Goates 1996). In particular, the polychlorobenzene 'hexachlorobenzene' (HCB) was so effective against common (Purdy 1955; Holton and Purdy 1954) and dwarf bunt that research into their integrated control came nearly to a standstill (Bruehl 1989). By 1957, the incidence of common bunt balls in UK wheat harvests had dropped from 33% to just 0.2%. In the same study, 0% of 122 1957 harvest samples were contaminated with common bunt spores (Marshall 1960). In 1966, the fungicide carboxin was released as a method of control

for bunts and smuts (Russell 2005) successful enough that it is still in use today under the trade names 'Vitavax®-200' and 'Vitavax®-34' (Wegulo 2009). By 1967 common bunt, which had ranked highly among the world's most destructive plagues and had been a textbook example of plant disease, no longer placed in the Top 7 disease threats due to the enormous success of seed treatment (Bruehl 1989).

However, seed treatment could not provide a total solution. While seed coating compounds proved very effective in most of the world, harvesting and threshing of bunted wheat contaminated agricultural soils heavily - causing lasting buildup of spores and intensifying infection pressure (Fischer and Holton 1957). Furthermore, common bunt remained a threat due to the improper use of fungicides (Goates 1996; Williams, Jr. and Gough 1984). Even in 1979, decades after the introduction of fungicides conferring near perfect control, common bunt caused 5-7% losses in North Africa and Central Asia (the ICARDA region) (Saari et al. 1996; Hoffman 1982). As the 1990s approached, fungicides began to occasionally falter even at label-recommended rates (Williams, Jr. 1988).

Notably, in 1974, the People's Republic of China issued a zero-tolerance policy for contamination of agricultural shipments with dwarf bunt spores. As previously discussed, many *Tilletia* species but most notably the common and dwarf bunt fungi share strikingly similar features and a complicating ability to hybridize with each other. Because common bunt was already present in China and was therefore not a trade barrier, it became exceedingly important to American wheat producers that *Tilletia* spores in their shipments be rapidly and precisely identified to species. This led to a flurry of research in the late 1970s and 1980s, with questions that are only recently being resolved (Forster et al. 2022). Practically, identification has always been intertwined with management. Common bunt, once identified in a region, can be controlled by a number of seed treatments. Dwarf bunt, on the other hand, requires more careful management and the application of a single protectant, difenoconazole, to seed. In the enmeshing of identification with global trade and American economic well-being, assays that were sensitive with a paucity of spores, simple, rapid, and requiring minimal training became necessary (Trione and Krygier 1977).

Recent Status of Common Bunt Distribution and Control (1990-present)

In the past three decades, control methods have included development and planting of resistant varieties, ensuring the use of clean seed, using broad crop rotations, and the utilization of chemical or organic seed treatments (Blöch 2021; Klaedtke et al. 2021; Carris 2010; Jones and Clifford 1983). These methods are in constant development, and the international scientific community continues work to better and more consistently manage common bunt outbreaks globally (Klaedtke et al. 2021).

Control may, in part, be afforded by good management of cultural practices; shallow sowing, sowing clean seed, and sowing in temperatures unfavorable for the germination of the fungus (Anderson Onofre 2020; Gaudet and Menzies 2012; Murray et al. 2009; Gaudet and Puchalski 1990), although these are not entirely effective (Waldow and Jahn 2007). For example, recommendations to plant into soils warmer than 16°C may avoid common bunt, but leave the plant vulnerable to barley yellow dwarf virus (BYDV) and be ineffective or detrimental for varieties that produce a shorter coleoptile in warm conditions (Anderson Onofre 2020). Additionally, sowing susceptible varieties later will not prevent or moderate infection relative to planting earlier (Gaudet and Puchalski 1990). Goates (1996) describes that crop rotations in the absence of seed treatment may be used. Although the spores may remain viable in herbarium specimens for decades (Fischer 1936), it is generally accepted that in field conditions they lose viability after about 2 years (Woolman and Humphrey 1924). In Italy, crop rotations are used mostly successfully. If common bunt is confirmed in a location, they will additionally mow down the crop and any wild cereals and treat all seed and farm tools with 2% sodium hypochlorite (Bussi et al. 2021).

In general, certified seed can be purchased to avoid seedborne contamination. Part of the success of bunt control is the ease of washing spores from seeds with chemical seed treatments (McNeil et al. 2004). For example, seed lots can be quickly washed with hot water or products such as ‘Tillecur’ making certified seed easier to produce and more readily available (Waldow and Jahn 2007). Seed certification programs can be very successful; in 1992 although spore contamination was moderate, only one bunt ball in one of the many certified seed samples studied was found (Cockerell and Rennie 1996). A major issue in common bunt control is the use of

contaminated farm-saved seed, which underscores the need for certified seed (Blöch 2021; Cockerell and Rennie 1996).

In several Nordic countries, testing of winter wheat seed for common bunt contamination is mandatory prior to planting (Brodal et al. 1997). If certified seed is not an option, phytosanitary analysis of the seed lot is conducted and if the spore load is over a nationally-defined (and potentially cultivar- and environmentally-modified) threshold, seed treatments are recommended (Matanguihan et al. 2011, Cockerell 1997, Brodal et al. 1997, Cockerell and Rennie 1996).

Currently, a range of chemicals are effective against both seedborne and soilborne common bunt inoculum. By the 1990s these treatments had become so successful that bunt "became so uncommon [in the UK] that most farmers were unfamiliar with the symptoms or damage [it] could cause" (Cockerell and Rennie 1996).

The history of the acceptance, success, and disavowal of different seed treatments is complex. In the mid 1990s the wildly successful and affordable organomercury seed treatments were widely banned for their toxicity to the environment and potentially humans (Brodal et al. 1997; Lisker and Klein 1997; Cockerell and Rennie 1996). From 1950 to the late 1980s, 'Caspan,' an organomercuric compound, was successfully used as the only wheat seed treatment in Israel. However, after its discontinuation, Israeli wheat production was further challenged by common bunt (Lisker and Klein 1997). In 1996, common bunt infections were on the rise globally because of host resistance that was no longer effective, the release of new cultivars without resistance genes, and "inadequate seed treatments," which lead to an increase in global soilborne inoculum (Saari et al. 1996). Tried and true hexachlorobenzene faltered, being both overcome in tests in Australia (Kuiper 1965) and presenting serious environmental concerns (Goates 1996). Although a 1997 survey of seed pathologists in Western Europe documented concerns that the replacement fungicides were not as effective at controlling common bunt (Cockerell 1997), as of 1996, carboxin, etaconazole, hexachlorobenzene, thiabendazole, triadimefon, triadiminol, and pentachloronitrobenzene were noted for their efficacy against both seedborne and soilborne inoculum. Triadiminol, marketed as 'Baytan', emerged as a product capable of more effectively controlling both seedborne and soilborne common

bunt - although close attention to application details is necessary to prevent adverse growth effects (Goates 1996; Gaudet et al. 1989; Wainwright and Morris 1989; Hoffman and Waldher 1981).

Of these, carboxin remains in use (Wegulo 2009) although it is inconsistently effective due to an inability to achieve target application rates or correct for the variability in the seed micro-environment (Gaudet et al. 1992). Triadiminol is also still in use, despite mild EPA concerns for avian and human health (United States Environmental Protection Agency 2006). Etaconazole is no longer approved for use by the European Union due to discovered toxicity to aquatic life with long-term effects (European Union 2009).

Hexachlorobenzene fell out of use as well after designation as a carcinogen, causal agent of liver disease, and threat to environmental health (International Programme on Chemical Safety (IPCS) 1997). Others currently out of or with limited use include thiabendazole, triadimefon, and pentachloronitrobenzene. Those controlling for only seedborne inoculum included benomyl, chloroneb, fuberidazole, maneb, pyrocarbolid, and TCMTB (Goates 1996; Gaudet et al. 1989; Hoffman and Waldher 1981). Although maneb has been banned in the EU and pyrocarbolid is not currently listed in PubChem, benomyl, chloroneb, fuberidazole, and TCMTB are still in varying degrees of use as of 2021. Copper oxychloride is currently used as a seed treatment in Italy to control common bunt (Bussi et al. 2021). Although tested only against seedborne inoculum, triazole was shown to provide excellent control as early as the 1980s (Goates 1996; Gaudet et al. 1989; Efthimiadis 1988). In the ensuing decade and a half, some of these have fallen out of use for various reasons but new products have come onto the market and common bunt remains at the mercy of a series of fungicides.

Additionally, difenoconazole ('Dividend®') was released as the only systemic fungicide that almost completely controls both seedborne and soilborne common and dwarf bunt (Goates 1996; Williams, Jr. 1991). As of 2009, Murray et al. reported that the most effective and widely used fungicides for controlling common bunt are seed treatments of carboxin, some benzimidazoles and difenoconazole. Carboxin (and fanaminosulf likewise) prevent germination of seedborne teliospores, which is a

common mode of action among the fungicides effective against the smuts and bunts (Kollmorgen and Ballinger 1987). Despite the success of carboxin, there is some evidence that these fungicides (along with maneb and others) vary in effectivity with environmental conditions (Gaudet et al. 1989).

There is, too, the constant concern that currently available modes of action in our seed treatment fungicides will be overcome by the quickly evolving pathogen. Although mitigation strategies to delay or remove the possibility of fungicide resistance have been described and recommended by many chemists (Hollomon 2015), these strategies are not always employed in bunt management. The example of a lack of reported mixture partners in 'Dividend®' was reported previously in this review, and the same lack seems to be shared by 'Allegiance®' products, 'Apron® XL,' 'Captan®' products, 'Charter®,' 'Dithane®' products, 'Dynasty®,' 'Grain Guard®,' 'LSP,' 'Manex®,' Manzate®' products, 'Maxim® 4FS,' 'Penncozeb®,' and 'Vitavax®' products (Wegulo 2009).

Although seed treatment for common bunt is routine in many countries, it is not generally used on farm-saved seed. When farm-saved seed is used in successive generations in more favorable conditions for common bunt, increases in common bunt incidence have occurred (Cockerell and Rennie 1996). This was the case in Eastern England in the early 1990s, where unusually dry weather and the potential development of a more persistent *T. caries* strain, facilitated unusually high levels of infection (Yarham 1993).

Seed treatments have been economically unviable in certain regions (Madenova et al. 2021; Yorgancılar et al. 2016), as this method of control comes at a price. In the late 1990s UK cereal growers spent an estimated £23 million on fungicide seed treatments to exclude bunts (Paveley et al. 1997). These costs have only risen, and not every nation facing common bunt is in an economic position to afford seed treatments at that scale. In the late 1990s, the lack of seed treatments in Kazakhstan resulted in 15-38% infection corresponding to one third of the yield being lost. Of the wheat remaining, much was rendered unusable for milling or feed (Madenova et al. (2021). In Serbia, during the UN embargoes of the 1990s, chemical seed treatments were not available and many were discontinued (Jevtić et al. 2021). In 2006, the Czech Republic

faced economic conditions that forced farmers to increase production of winter wheat in organic and low-input systems - increasing the spore loads of common bunt in the fields and incidence of common bunt in the grain (Váňová et al. 2006). In Eastern Europe prior to 2010, economic stress made seed treatments difficult to afford, forcing many producers to replant their own potentially contaminated seed (Zouhar et al. 2010). Currently, many countries in North Africa and Central Asia lack access to seed treatments – there only 40% of seeds may be treated for common bunt, resulting in infections in 5-7% of the crops (Madenova et al. 2021). The costs of seed treatments are rising relative to their past financial impact even still (Madenova et al. 2021).

Races of the fungus are monitored routinely to aid in the understanding of host resistance genotypes necessary for certain regions (Christensen and Borgen 2021a; Jevtić et al. 2021; Orgeur 2021; Ehn et al. 2021; Matanguihan et al. 2011). In 1996, noted bunts and smuts researcher Blair Goates wrote that "the development of bunt-resistant cultivars may be the best method to control the disease when resistant sources are available," acknowledging the effectiveness of fungicides but ranking them lower than resistance for the potential environmental, health, and availability problems associated with chemical control. Most resistant cultivars that are released are not assessed for their specific resistance genotype (Gaudet and Puchalski 1989)**,** though analysis of heredity and genetic inheritance can provide some insight.

Occasionally, germplasm screenings are performed. For example, studies of bunt populations in the US and Europe over several years resulted in the understanding that European bunt populations were avirulent to *Bt5*, *8, 9, 10* and *11* and generally virulent to *1, 2, 3,* and *7.* US bunt races were avirulent to *Bt8*, *11*, and *12* and only five races were virulent on either *5, 9,* and *10* (Matanguihan et al. 2011; Goates 1996). In 2016, scientists reported the effectiveness of *Bt8*, *Bt9* and *Bt10* against the local races in Austria (Madenova et al. 2021; Hagenguth 2016) Another 2016 study of common bunt collections in Iraq revealed that the local races were avirulent to *Bt3*, *Bt5*, *Bt6*, *Bt9*, *Bt11*, and *Bt12* (Madenova et al. 2021; M. Al-Maaroof et al. 2016). Nebraskan races were avirulent to *Bt6*, *Bt9*, *Bt12*, *Bt13*, *Bt15*, and *Btp* as of 2018 (Mourad et al. 2018). Observations in 2021 indicated that a new race of *T. laevis* has developed in Sweden,

virulent to the 'Stava' wheat cultivar that likely carries *Bt8* and *Bt9* (Christensen and Borgen 2021a).

Resistance screening is an important part of resistance breeding, and studies to screen for resistance have been developed in Europe (mostly under organic systems) and the U.S. (mostly under conventional systems) (Matanguihan et al. 2011). In these screenings, resistance is almost always calculated by a percentage of smutted heads. Goates (2012) set a threshold of 10% infected heads when evaluating the reaction of spore mixtures. Classically, in this system avirulence is characterized by a <10% bunting of heads. Meanwhile, anything greater than the 10% threshold is considered virulent. These thresholds were narrowed by Madenova et al. (2021), allowing only 5% or less infected spikes for classification as a resistant variety. Their team reintroduced the distinction between intermediate (6-25%), susceptible (26-50%), and highly susceptible (51-100%) varieties as well. Despite the stringency and specificity in this new classification system (yet to be fully adopted), the discussions of Pope and Dewey (1975) on the failures of rating disease at heading remain unanswered. They posited that, based on the nature of potential quantitative resistance and the importance of less prominent resistance phenotypes such as diminished stands and changes in tillering, this may fail to account for "degrees of successful opposition of wheat... to development of the smut pathogen." Percentage of smutted heads also fails to account for the complexities of the reaction between virulence genes, genes that modify those genes, pathogen fitness, and the environment (Thomas 1991).

Prior to the rise of PCR-based detection methods, the only way to confirm a common bunt infection was time intensive lab procedures involving a seed wash, filtration onto a cellulose nitrate filter, and microscopic examination for spores that must be carefully separated from debris, as described by Cockerell & Rennie's (1996) protocol**.** Although the assay could be performed in 24 hours, it was labor-intensive and relied on expertise in identification of the spore morphology (Roberts et al. 2007; McNeil et al. 2004). This further complicated integrated management techniques, particularly resistance breeding.

Scientists are working on methods and beginning to successfully employ molecular markers to hasten the screening process and begin to answer the

shortcomings of classical resistance screening. While field trials are time-consuming and subject to variability in low infection years, molecular markers for known resistance genes can be applied early on in the wheat's life cycle (Madenova et al. 2021; Matanguihan et al. 2011). For example, Madenova et al. (2021) used previously published SSR (Steffan et al. 2017) and SCAR (Laroche et al. 2000) markers to determine the available resistance genes 5 days post-inoculation in 61 bread wheat varieties available in Kazakhstan. This screening for specific resistance genes using these markers in the available germplasm, a process called "marker-assisted selection (MAS)," will then be used to select parental varieties and assess progeny for the presence of the same resistance genes that are most effective against the bunt races present in Kazakhstan. In this way, molecular markers can support the most time- and cost-effective efforts to produce the most adapted varieties to different wheat-growing regions. The use of such markers can potentially be supplemented or replaced by Genomic Selection (GS). In a dwarf bunt study, MAS and GS were compared to evaluate the ability to support breeding efforts involving minor quantitative resistance genes (Krause et al. 2021). As will be discussed in detail later in this literature review, major effect *Bt* genes have been hypothesized to be supplemented by minor genes that can ostensibly confer greater and more durable resistance. Utilizing these will be important in the years to come, but Krause et al. (2021) found that MAS alone cannot identify these minor genes where GS can. Both MAS and GS are currently in preliminary use in Vienna, Austria (Ehn et al. 2021). Ehn et al. (2021) have been developing novel experimental lines using both KASP markers and QTL through MAS for bunt resistance, as well as genomic assisted background selection to improve agronomic traits.

Despite these advances in breeding, the adoption of resistant varieties is not always commonplace in locations where common bunt is endemic (Matanguihan et al. 2011, Wolfe et al. 2008). Out of 12 commonly planted lines in Serbia and Montenegro, only 4 are resistant (Rajković and Dolovac 2006). Of the 26 cultivars registered in Lithuania, 2 had moderate resistance and none were highly resistant (Liatukas and Ruzgas 2005). Although Madenova et al. (2021) note that over 200 commercial varieties and promising breeding lines demonstrate a high resistance to common bunt in Central Asia and the Caucasus, they also report that practically none of them are

actually in production. This may, in part, be due to the lack of active selection for resistant varieties in current breeding programs, as in the case of soft kernel durum lines. In the search for durum lines with better general milling quality, researchers bred soft-kernel durum varieties and tested them against common pests of wheat including dwarf bunt. Although not bred for resistance, initial research found excellent dwarf bunt control by cv. 'Svevo' progeny. However, the lines still lacked adaptation to common subpar soil types so the authors agreed that further breeding efforts would be necessary to improve the marketability of the grain. The resistance itself was seen as a rarity, given that resistance to common and dwarf bunt is highly atypical without active selection in the breeding program (Kiszonas et al. 2019).

Control in organic wheat faces challenges that continue the historic battle between man and bunt as those fungicides that have been developed are generally not permitted in certified organic systems. Although organic systems are able to reduce disease pressure from diseases such as powdery mildew and *Septoria tritici* as opposed to conventional production, organic systems are much more sensitive to even mild disease pressure from the bunts (Wolfe et al. 2008). Organic farmers must therefore rely on clean seed, cultural practices, non-chemical seed treatments, and host resistance. In the 2010s, focus in organic agriculture was placed on hot water treatments, heat treatments, and plant-based substances to control common bunt. Control has also been pursued through biocontrol strategies and microbial volatiles (Matanguihan et al. 2011). 'Tillecur', a yellow mustard powder product, has been approved for organic production and is reasonably effective (Matanguihan et al. 2011), though it should be noted that it varies in efficacy and increases production costs significantly without being applicable on a large scale (Lammerts van Bueren et al. 2011). Alternative organic seed treatments including skimmed milk, hucket (a type of skimmed milk local to North Africa), and wheat flour have been investigated in trials in North Africa and West Asia, with ~95% reductions in bunt incidence shown in both milk treatments. It is unclear whether the milk suppresses the pathogen or encourages competition among soil microbes or any number of other possibilities, but the efficacy was consistent across space and time (El-Naimi et al. 2000). Isolate MA 342 of the bacteria *Pseudomonas chlororaphis* has been found to inhibit common bunt well in the

field (Johnsson et al. 1998; Hökeberg et al. 1997). Since that discovery, the strain has been made commercially available as the biopesticides 'Cedemon' and 'Cerall'. 'Cerall' is more applicable to common bunt, and is registered in Austria, Finland, Lithuania, Sweden, Switzerland, and the United Kingdom, likely among other countries (Matanguihan et al. 2011). Host resistance, though, is the "most effective, economically feasible and environmentally sound control method" despite the relatively quick eventuality that race-specific resistant lines will be overcome by new pathogenic races (Matanguihan and Jones 2011; Matanguihan et al. 2011).

Resistance breeding in organic agriculture is underfunded and less explored than conventional breeding programs, and often considered unnecessary by conventional breeders (Wolfe et al. 2008). Organic breeders do have a limited selection of resistance sources in breeding lines that they can work with and, according to Gladysz et al. (2021), breeding for organic agriculture has "already been achieved and will continue with further registrations in the near future." For example, *Bt10* has been bred into cultivars 'Tillexus' and 'Tillstop.' However, testing these cultivars with such a limited resistance genotype against individual local races of common and dwarf bunt in Austria so they could not be recommended fully to farmers in that region. Breeders have been able to use this information, with the aid of a little luck, to develop and promote cultivars such as 'Tillsano' that have more broad-spectrum resistance in organic conditions. Breeding continues with the development of varieties for organic agriculture that combine race-specific and non-specific resistance modes to produce durable resistance (Gladysz et al. 2021).

Although several resistant varieties bred for organic systems have been released, resistant varieties bred for conventional systems while greater in number may lack the qualities necessary for survival in lower-input and organic agriculture (Lammerts van Bueren et al. 2011; Löschenberger et al. 2008; Wolfe et al. 2008). With selection under conventional systems, the plants may not have the necessary traits required for lower-input systems with less crop protection (Wolfe et al. 2008; Murphy et al. 2007; Lammerts van Bueren et al. 2002). Additionally, plants bred with the intention of being chemically protected from diseases will likely be susceptible to those diseases without the protectant due to lack of selection during the breeding process

(Lammerts van Bueren et al. 2011). Modern conventional breeding programs are largely a testament to this phenomenon. Of the many wheat breeding programs in Europe where most commercial varieties are highly susceptible to common bunt, conventional breeders have "no interest In breeding for resistance to [common and dwarf bunt]" likely because they are conventionally controlled (Wolfe et al. 2008).
In northern and western Europe, control of common bunt is beginning to falter as the culture shifts towards organic production (Matanguihan et al. 2011, Brodal et al. 1997). Although European authors such as David Blöch (2021) have astutely observed that common bunt is not *only* an issue in organic agriculture, as of 2004, new regulations went into effect in Europe which required that wheat labelled as organic must be grown from organically produced seed. This may result in shortages, as the lack of protection against common bunt has reduced the availability of organic seed in some years (Lammerts van Bueren et al. 2003). While Blöch discusses the necessity of using clean seed not farm-saved, if clean organically produced seed becomes scarce than reliance on farm-saved seed may continue to be a problem.

Europe, in addition to being a hotspot for organic agriculture, is also a continent where common bunt is highly endemic. In the 1990s, the UK, Germany, Denmark and (to a lesser extent) Sweden and Norway saw a resurgence of common bunt infections that was attributed to inoculum buildup in the soil (Cockerell 1997; Brodal et al. 1997). Testing in the UK showed that 20-60% of farm-saved and certified seed was contaminated with common bunt (Cockerell and Rennie 1996), and most organic seed lots bore significant spore loads of common bunt (McNeil et al. 2004). As of 2008, due to the combination of rapid pathogen evolution, improper use of fungicides, and heavy reliance on monocultures, outbreaks of common bunt have become more prevalent in Romania (Fraga et al. 2008). A 2021 conference presentation described the incidence of common bunt in Serbia rising even in conventional systems. Of the 151 seed samples studied, 74% were contaminated with common bunt spores below the saleable threshold of 0.1 teliospore per seed and 4/16 commercial samples harbored more than the threshold amount (Jevtić et al. 2021).

At this point, however, the epidemiology of the disease is such that, as acceptability of seed treatments wane and their functionality and availability is

threatened, the fungus is poised to "cause economic devastation to low-input and organic farmers" (Matanguihan et al. 2011). Many have concluded, based on their work with common and dwarf bunt, that these diseases have great potential to infect and contaminate seeds at an increasingly higher level (Jevtić et al. 2021). There is reasonable fear, given the hybridization potential of common bunt fungi with each other and other *Tilletia* species, that the genetic variability in bunt populations is increasing faster than we are developing resistant varieties and chemistries. It's possible, even, that the fungi may be evolving resulting in an expansion of the host range and geographic influence (Pimentel et al. 2000). With the prominence of organic ideology in Europe, common bunt will need additional attention unless a more highly effective organic seed treatment is quickly developed, or existing resistant varieties last an unusually long time. In general, Murray et al. (2009) acknowledge that, given the gene-for-gene race-specific nature of resistance breeding, resistance should be combined with fungicide use in areas with high levels of soil-borne inoculum. Additional work has highlighted the necessity of good agronomic practices and wide crop rotations in conjunction with efforts to ensure clean seed (Blöch 2021). These calls for integrated approaches using resistant varieties along with chemical and biological control methods have been echoed by many researchers, especially given the economic and evolutionary difficulties with using chemicals alone and the slower pace of breeding (Madenova et al. 2021).

Infection Cycle and Histopathology

Common bunt spore loads may build up in the soil of fields planted with untreated seed of susceptible host plants (including, barley, which may also host the fungi) (Saari et al. 1996). Initial infections, however, most frequently develop from seed contaminated during harvest during the previous growing season. In the wheat head, intact common bunt sori prevent teliospore germination by producing or inducing trimethylamine, as well as several other secondary compounds (Goates 1996; Ettel and Halbsguth 1964). During harvest, sori are broken open and spores contaminate the surface of the seed. Historically, highly infected crops caused clouds of spores to waft over to adjacent fields. When contaminated seeds are planted the next year, disease may develop if

favorable conditions are present and the seed is sufficiently coated with spores, which is more likely to be due to seedborne inoculum than soilborne (Murray et al. 2009; Wegulo 2009; Goates 1996; Saari et al. 1996). In a laboratory environment, it takes 35,000 to 150,000 spores per individual wheat seed to guarantee infection (Holton and Heald 1941).

Given that teliospore germination is most successful in cool and moist conditions, sowing seeds deeper (i.e. 7 cm. vs. 4 cm.) seems to increase the incidence of infection by providing a more favorable environment for teliospore germination and supposedly delaying the development of the crown node long enough to give the pathogen time to infect it (Holton and Heald 1941). Factors such as temperature, moisture and acidity affect the germination of common bunt spores and the infection process. For example, different races of common bunt fungi are more or less successful at infecting the same cultivars at different planting dates (Gaudet and Puchalski 1990; Kendrick and Purdy 1962; Kendrick and Holton 1961). Additionally, clay, sandy, or acidic soils inhibit germination of the spores. The fungi prefer a "mineral soil with a clay base, with plenty of humus content, and neutral in its reaction" (Fischer and Holton 1957). Infection is more likely to occur at cooler temperatures, sometimes even cooler than those optimal for the fungus, supposedly due to the delayed growth of the plant. It has been postulated that this inability to rapidly elongate allows the cool-tolerant fungi to spread more rapidly into growing point tissue than the plant can elongate. However, experiments by Rodenhiser and Taylor (1943) investigating the infection of plants at different day lengths found plants that grew more rapidly showed higher disease incidences (Rodenhiser and Taylor 1943). If "outpacing" were an explanatory phenomenon, this would not have occurred (Fischer and Holton 1957).

Teliospores are released from the sorus, which then germinate within 70 hours and produce infection hyphae roughly 4-10 days after seeding (Wegulo 2009; Saari et al. 1996; Swinburne 1963). Germination is not uniform, and multiple infection events can occur. In a laboratory setting, infection events proceeded from 5-21 dpi and as many as 40 infections may occur on a single coleoptile (Gaudet et al. 2007). In 1963, Swinburne observed "numerous" penetration points on coleoptiles after 9 days, which only increased after 11 days (Swinburne 1963).

Germination begins with the promycelium (basidium) splitting through the spore wall's hydrolyzed area (Dastur 1921) and growing to different lengths in an environmentally-dependent manner. Typically 8-16 primary sporidia (though a range of 4-16 have been observed (Sartoris 1924; Dastur 1921)) then grow through the promycelium to form a whorled compact bundle at the tip of the promycelium (Murray et al. 2009; Goates 1996; Kollmorgen et al. 1978; Dastur 1921). The cytoplasm then migrates from teliospore to the promycelium and finally into the primary sporidia, with septations forming behind it as it moves (Goates 1996; Goates and Hoffmann 1986; Sartoris 1924). In any given bundle of sporidia, the mating types (+) and (-) are present. The sporidia form short conjugation pegs, which allow pairs of opposite mating types to fuse together to form an H-body (Kollmorgen et al. 1979, 1978). Without both mating types present, fusion will not occur and the pathogenic dikaryophase will not form (Goates 1996; Kollmorgen et al. 1979; Fischer and Holton 1957; Flor 1932, 1931), however those primary sporidia that do not mate may still produce secondary sporidia or mononucleate hyphae (Kollmorgen et al. 1979, 1978; Fischer and Holton 1957; Holton and Heald 1941).

The fused primary sporidia then form either secondary sporidia (allantoid or filiform), vegetative hyphae, or infection hyphae (Goates 1996). Secondary sporidia are forcibly discharged (if allantoid) (Goates and Hoffmann 1986) and go on to form either additional sporidia, vegetative hyphae, or infection hyphae (Goates and Hoffman 1979; Sartoris 1924). All of these are visible on the surface of the inoculated coleoptile. Swinburne (1963) found "abundant promycelia, primary sterigmata and hyphae, secondary basidiospores and hyphae," although none were found in association with the penetration points, likely due to the limitations of the microscopy methods available to him at that time.

The infection hyphae (sporidia) then infect the plant host in the soil, provided the plant is less than 12 days old (Sartoris 1924). After 4 days, the seed pericarp is found to be colonized and, after 7 days, the coleoptile is infected (Swinburne 1963). The spores germinate and primarily infect the coleoptile (Hansen 1958; Woolman 1930), occasionally growing along the surface before locating an appropriate penetration point (Sartoris 1924). Penetration is more commonly observed at the intersection of 2

epidermal cells (Chen et al. 2021; Murray et al. 2009; Gaudet et al. 2007; Goates 1996; Woolman 1930; Sartoris 1924; Dastur 1921) quickly following germination and before the seedling emerges (Wegulo 2009; Saari et al. 1996; Goates 1996). Penetration may be aided by the formation and action of an appressorium (Jones and Clifford 1983; Churchward 1940) or multiple appressoria per hyphae (Churchward 1940). This entry into the host is forceful, and the epidermal cell walls may rupture (Dastur 1921). After penetration, the external portion of the fungus quickly withers away (Sartoris 1924).

Multiple germinated spores will produce multiple sites of infection, with the number of infections correlated to the severity of bunting in the heads in susceptible varieties (Gaudet et al. 2007). In 1963, Swinburne reported increasing numbers of penetration points on the coleoptile that induced shriveling in the plant tissue. *T. caries* has also been observed to produce multiple appressoria per hyphal thread (Churchward 1940) and infect at multiple points along a single fungal hypha growing in the indentation between epidermal cells along the axis of the coleoptile (Swinburne 1963). The pathogen mycelia may mat the surface of the coleoptile (Churchward 1940). In the beginning of infection, enzymatic dissolution of the middle lamella between wheat epidermal cells can be observed (Gaudet et al. 2007). Chen et al. (2021) observed that at germination, hyphae start from “small tips” and form a hyphal network to colonize leaf and root cortical and root rhizodermal cells intercellularly. This infection was much more developed in the susceptible cultivar ‘Dongxuan 3’ than the more resistant cultivars also infected with *T. controversa*.

Woolman (1930) investigated side-by side comparisons of inoculated susceptible (Hybrid 143) and resistant (Turkey Wn. 326 × Florence) wheat lines. He found that even as the mycelium progressed in the susceptible Hybrid 143 into the first true leaf, then the second leaf sheath, then the subsurface internode, and eventually was “extensively and profusely distributed through the tissues of all the leaves and in the axis of the plant and... growing point” - the infection in the resistant Turkey Wn. 326 × Florence progressed no further than the first or second leaf sheath if it left the coleoptile at all. The fungus then systematically infects the plant, “emanating in all

directions" both inter- and intra-cellularly (Gaudet et al. 1993; Swinburne 1963; Hansen 1958; Woolman 1930).

Classic studies reported that, after first penetrating the coleoptile, hyphae quickly colonize the first leaf base, then the second, moving through sequential leaf bases or down the leaf base to the tissues directly beneath the apical meristem. Hansen (1958) reported that throughout the growth through the plant, the hyphae were found in sclerenchymatous tissues, but only in older tissue whereupon they are closely appressed to the cell wall and partially devoid of cytoplasm - apparently dissolving as the fungus spreads to newer tissues. Using scanning electron microscopy, Ren et al. (2021b) found malformations in mesophyll cells of susceptible cultivar 'Dongxuan 3' infected with an unspecified race(s) of *T. caries* at Zadoks 11 (first-leaf stage). They also reported seeing hyphae in the roots and leaves at this stage, although they did not see resultant deformation. Hansen (1958) found the fungus first in the leaves and shoot axis in these early stages. Ren et al. (2021b) continued on to study later growth stages. At the two-leaf stage, they found cellular deformation surrounding sieve tubes.

Hansen's work was very thorough, and an English summary was provided, but the manuscript was only published in German which was unfortunately inaccessible to the author. The process was thoroughly detailed in English by Swinburne in 1963, in which two varieties of susceptible wheat plants, the then-popular 'Fylgia' and 'Capelle Desprez', were studied with an unknown race or race mixture of *T. caries*. Swinburne found that:

- the 2nd leaf stage had inter- and intracellular hyphae in the coleoptiles, though not in the leaf bases or seed tissues of 'Fylgia'. In 'Capelle Desprez' the first leaf base may be colonized, and hyphae were only intracellular until the coleoptile browned and shriveled.
- At the 3rd leaf stage, the fungus had spread to the 1st leaf base (and second in 'Cappelle Desprez') and changed the staining pattern of infected cells compared to uninfected cells.
- The 4th leaf emergence coincided with infection of both the 1st and 2nd leaf bases and the cortex of the mesocotyl, with some plants also infected to the 3rd leaf base. The nuclei of infected cells stained differently than those of uninfected

cells. At this stage, the crown-node reached the soil surface. In 'Capelle Desprez', the infection was more prominent in the 3rd and fourth leaf bases and found in some plants directly beneath the growing point.

- At the emergence of the 5th leaf, the lower internodes began to elongate and the hyphae that had been found in the 1st and 2nd leaf bases (lower 3-4 mm of the plant) had disappeared, though holes were present suggesting that the hyphae degraded as it continued to colonize newer tissues including the 4th and 5th leaf bases, 6th leaf primordium, the tiller buds, and less commonly at the growing point.
- At the 6-leaf stage, with the flag leaf present, hyphae were present in the flag leaf base as well as random, individual hyphal colonization of unpatterned flower primordia without visible infection of the rachis.
- At the emergence of the ear, hyphae were found only in the carpels and chlamydospore formation started, leading to the beginning of spore masses replacing the ovuliferous tissue.
- Once the seeds had swollen sufficiently, the bunted kernels were distinguishable from healthy heads. Heads were not uniformly infected, and the infection level in individual heads varied greatly, with hyphae still present in only the last internode and sometimes the 2 nodes closest to the last and chlamydospores occasionally found in the node pith and cortex.

Work by scientists in 2021 investigated the Zadoks 13 (third leaf) growth stage using high-powered microscopy techniques including scanning electron microscopy (Xu et al. 2021; Ren et al. 2021b), transmission electron microscopy (Xu et al. 2021) and laser scanning confocal microscopy (Ren et al. 2021b). Ren et al. (2021b) found that the membranes of the mesophyll cells were ruptured at the third leaf stage. Xu et al. (2021) compared the tillering stage to the third leaf stage and found that the plant was more fully colonized by *T. controversa* in the susceptible variety 'CU42' at the tillering stage than at the third leaf stage, although they did see some deformities in the stem (meristematic) cells and mesophyll cells (Xu et al. 2021). In comparison to resistant varieties that showed no deformity in the root cells, stem cells or mesophyll cells, the susceptible were much more damaged.

At the tillering stage, beyond rupturing, mesophyll cells were deforming and their plasma membranes breaking (Ren et al. 2021b). Hansen (1958) noted that as the internodes develop the fungus is then found in the nodes and not the basal part of the plant. While Swinburne (1961) reported that, at the 4th-5th leaf stages (which likely coincide with the tillering stage) the hyphae were carrying along with the meristematic tissue and degrading the earlier hyphae, more aggressive colonization was reported in January 2021 in susceptible plants infected with *T. controversa* (Xu et al. 2021). Although the research team did not report the race(s) of *T. controversa* used or the genetic background of resistance in their studied cultivars, they found evidence of *T. controversa* in the roots, stems, and leaves in susceptible varieties 'CU42' and 'Dongxuan 3' at the tillering stage (Zadoks 21) using scanning electron microscopy and transmission electron microscopy paired with molecular qPCR detection. Their microscopy techniques provide a much higher resolution in images of fine morphology than sectioning and staining (Ren et al. 2021b), and it is likely that these results are more accurate than those obtained with light microscopy in the 1950s and 1960s. Additional confirmation of plant infection with the detection of a specific band on a gel from extracted leaf DNA, and analysis in comparison to resistant and mock-inoculated plants supported their results. Specifically, their results indicated that susceptible varieties had more severely damaged root epidermal and vascular bundles, more extensively deformed stem (meristematic) cells, more slightly deformed and shrunken, scattered mesophyll cells, more damaged root cortical bundle cells and cell contents, and significantly deleteriously changed nuclei, chloroplasts and mesophyll cells than resistant cultivars 'Mianyang 26/Yumai 47' and 'Yinong 18/Lankao 8.' They further reported that, in the susceptible cultivar 'CU42,' infection ruptured the stem cells, deflated and deformed the chloroplasts, and ruptured the nuclear envelopes within cells. These results indicate that, contrary to previous dogma, the *T. controversa* colonizes the plant extensively throughout at least the tillering stage.

Supporting this work, a presentation at the *2021 Virtual Workshop on Smuts and Bunts* in May reported that at the tillering stage, *T. controversa* has been found to leave a path of destruction. In the stems and leaves of susceptible variety 'Dongxuan 3' mesophyll cells, and the nuclei and chloroplasts of observed cells were "significantly

changed" and "more severely affected" than those tissues of resistant cultivars (Ren et al. 2021a). They explored the effects of dwarf bunt on root physiology in the susceptible wheat cultivar 'Dongxuan 3'. Morphologically, they found that *T. controversa* more severely damaged epidermal and vascular bundles, stem cells, and cortical bundle cells (including their cell contents) (Ren et al. 2021a). Although Ren et al. (2021a) do not specify in their conference abstract which resistant varieties were studied and what their resistance genetics might be, nor the races of dwarf bunt used, the results are still part of a novel growing consensus that *Tilletia* bunts are systemic and colonize roots. These results are likely applicable to at least some races of *T. caries* and *T. laevis* in concert with some wheat cultivars.

Xu et al. (2021) also studied resistant varieties to compare to susceptible varieties at the same stages; Zadoks 13 and Zadoks 21. Resistant variety 'Mianyang 26/Yumai 47' was found to have minor *T. controversa* colonization of the root cortical parenchyma cells at Zadoks 21, but no damage to stem cells at the first internodes above the roots and maintained cell structures. Resistant cultivar 'Yinong 18/Lankao 8' showed little difference at all between their roots and mock-inoculated roots, though, and the stem cells were entirely unchanged. Their work more broadly indicates that resistant wheat varieties likely mount a stronger, unknown defense mechanism between Z13 and Z21 to prevent hyphal expansion and cell degradation. It also more broadly implies that the plant defenses in susceptible cultivars may be weaker than previously understood, and allow for inhibition of standard plant function. This is puzzling given the lack of macro-phenotypic differences between infected susceptible cultivars and infected resistant cultivars throughout the tillering stage.

By the jointing stage (Zadoks 31), the mesophyll cells were similarly damaged and furthermore chloroplasts were misarranged and scattered throughout cells (Xu et al. 2021). This pattern was also identified by Ren et al. (2021b) in a *T. caries* infected cultivar, noting that chloroplast deformation could greatly impede plant energy accumulation. It remains unclear how the plants overcome this and display a healthy appearance throughout the jointing stage.

After the jointing stage, the fungus spreads to the developing head, lying latent until the ovaries begin to develop at which point the hyphae replace the young ovary

tissue, eventually producing teliospores (Murray et al. 2009; Wegulo 2009; Goates 1996). At maturity, Hansen (1958) found that the fungus was present in a majority at the head in susceptible cultivars, and much less so in its previously colonized tissue. The ovarian tissue and developing seed are rich in protein, fats and starches - providing the fungus with both the nourishment and protection to fruit (Carefoot and Sprott 1967). Ren et al. (2021b) found hyphae in leaf blades, leaf sheaths, stems, and glumes at the mature stage using transmission electron microscopy. They noted accumulation of hyphae in mesophyll cells, sieve tubes, and sieve tube companion cells. This accumulation of hyphae thickened infected cell walls. Interestingly, they also reported that, using scanning electron microscopy, teliospores were found, in addition to massive accumulation in ovarian tissue, in roots, stems, leaves, glumes, and awns of *T. caries* infected susceptible plants at maturity. The development of pollen is also diminished in infected susceptible plants (Chen et al. 2021).

The understanding of anther and ovary colonization by the closely related dwarf bunt fungus, *T. controversa*, was recently developed by Muhae-Ud-Din et al. (2020) using a modern method involving the injection of the wheat plant with germinated dwarf bunt spores at the early boot stage, and Chen et al. (2021) using the same inoculation method. Although this method does not represent colonization as in field infections, Muhae-Ud-Din et al. (2020) were able to observe that infection differentially reduced anther length, increased callose deposition, and modified ovarian tissue by fungal action between a resistant and susceptible cultivar. In the susceptible cultivar, anthers were significantly longer and wider after inoculation than in the inoculated resistant variety. Callose deposition was increased in the inoculated resistant cultivar over the inoculated susceptible cultivar, and the ovarian tissue was only colonized and modified in the susceptible cultivar. After 10 days, there was some observable hyphae in the resistant anther, but this did not result in disease. Chen et al. (2021) observed the epidermis, endothecium, and tapetum cells of the anther more specifically with confocal microscopy, finding that while no hyphae were present in the resistant cultivar, the same cells in the susceptible cultivar were heavily colonized. In the ovary, Fischer and Holton (1957) observed binucleate hyphae in the initial infection, but as the true teliospore is produced they were uninucleate and diploid. These hyphae were observed

in both epidermal and sub-epidermal cells of the ovary progressing to the formation of teliospores in the susceptible cultivar studied by Muhae-Ud-Din et al. (2020), but not in the resistant cultivar. It is worth noting that Muhae-Ud-Din et al.'s (2020) study only investigated one susceptible and one resistant cultivar with an unknown genetic background of resistance and challenged them with one unspecified race of dwarf bunt so their results may not be applicable to other cultivars and races, or necessarily to common bunt infection. However, their study was careful and well-reported. Chen et al. (2021) added a moderately resistant cultivar, but also did not specify the resistance genetics of any of their cultivars or the *T. controversa* race mixture.

According to Gaudet et al. (2007) and Swinburne (1963), if hyphae fail to colonize sequential leaf primordia and the apical meristems before internode elongation, disease will not develop. However, disease will necessarily develop if the hyphae do reach the growing point. Given that the infection must be successful at the beginning of internode elongation, which occurs over the span of only several days, it is unlikely that later stages of wheat growth would be able to moderate an infection.

Host Resistance

Host plant resistance likely originates from "a clear center of concentration ... extending from Serbia and Montenegro through Macedonia, Turkey, and Iran with the highest frequency of resistance occurring in Kosovo province in Serbia and Montenegro (36%) and Bakhtaran province in Iran (40.8%)" (Bonman et al. 2006). The origins of the resistance genes correlate with the geographic origins of wheat production, suggesting a long battle between the pathogen and the host.

Resistance is mostly deployed in breeding programs as a qualitative gene-for-gene response; a system in which "for each gene that conditions reaction in the host there is a corresponding gene in the parasite that conditions pathogenicity" (Flor 1971). Therefore, the resistance phenotype is controlled by a series of resistance genes (named *Bt1* through *Bt15*, and an additional gene *Btp*) for which specific alleles produce proteins which interact with specific genes and their effectors in different races of the bunt organisms in an environmentally-independent manner (St. Clair 2010; Kearsey and Pooni 1996). Accepting the gene-for-gene hypothesis, monogenic wheat lines are

used to characterize different races of common and dwarf bunt through the compatibility or incompatibility with the different resistance genes (Goates 2012; Flor 1971). These monogenic lines have not all had their specific resistance gene fully characterized, but because of the nature of major resistance genes their genotypes may be inferred from their phenotypes (St. Clair 2010). The different pathogenic races of common bunt (and the closely related dwarf bunt) are almost genetically indistinguishable variants that, presumably guided by currently undetected genetic differences, can be distinguished by their ability to attack different host resistance gene profiles. This interaction has classically been considered a gene-for-gene response, limiting wheat resistance to those bunt races that are not virulent to the particular resistance gene(s) possessed by the host (Goates 1996; Matanguihan and Jones 2011).

With only 16 resistance genes currently identified, the genetic basis for resistance is quite limited. For comparison, more than 60 resistance genes have been identified in wheat to protect against stem rust (*Puccinia graminis* f. sp. *tritici* Eriks. & E. Henn) (Megerssa et al. 2020). Given that most of the wheat cultivars grown as of 2011 are susceptible to one or more races of common bunt, this dearth of resistance genes is particularly troubling (Matanguihan et al. 2011). In the US, as of 1982 fewer than 12 bunt resistance sources were available, and likely the genetic basis of that resistance is even more limited (Hoffman 1982). Beyond the paucity of resistance genes currently identified, of which only 14 are effective across environments, we also face a lack of knowledge regarding those resistance genes and many other factors in host resistance (Muellner et al. 2020b). In other pathosystems, "monogenic" differentials selected by trial and error without specific knowledge of their genetics have been found to possess multiple resistance genes or a level of multi-gene resistance ('horizontal resistance') (Flor 1971).

Initial infection occurs both in resistant and susceptible varieties of wheat (Gaudet et al. 2007; Swinburne 1963; Hansen 1958; Woolman 1930) but progresses only if "complementary virulence genes of the pathogen exist for all the resistance genes of a particular host" (Goates 1996). Gaudet et al. (2007) postulated that the *Bt1* and *Bt7* resistance genes were present in the Turkey Wn. 326 × Florence variety that

Woolman (1930) studied. Woolman concluded that in a pathosystem of Turkey Wn. 326 × Florence with some race(s) of common bunt, due to the observed spread of the mycelium into the first true leaf, the plant initiates suppressive action "about the time the mycelium passes into the first true leaves." In resistant varieties, the hyphae generally do not progress to the apical meristem of resistant plants (Gaudet et al. 2007; Swinburne 1963; Hansen 1958; Woolman 1930). In resistant plants, the mycelium is apparently confined to the coleoptile where callose deposition may exclude further infection (Gaudet et al. 2007). Woolman (1930) noted that the bunt fungus "ceases to be a menace" in resistant varieties after 60 days of plant growth. Even in susceptible plants, the pathogen mycelium has been found to reach the growing point in only about 50% of plants studied in under 50 days (Hansen 1958).

Phenotypes of disease have also been relatively underreported, especially in the context of their significance to the nature of resistance. While typically "resistant" cultivars are those showing less than 10% infection in spikes by a specific race (Goates 2012), Pope and Dewey (1975) thoroughly cataloged the range of disease phenotypes observed in their extensive work with dwarf bunt. Their conclusion of the plant's ability to perform "increasing degrees of successful opposition" to dwarf bunt are supported by observations of different outcomes of infection. In the most susceptible genotypes, they observed reduced stands and tiller heights, as well as weakening and collapse of the infected plant. In less susceptible plants, tillering is increased but the tillers are shorter. In cultivars with the *Bt3* resistance gene, foliar symptoms develop as mottling and speckling. These symptoms seemed, to Pope and Dewey, as though the fungus is creating a toxin affecting the host plant. Some of these mottled plants, however, recover before heading and show only 50% smutted heads, which are confined to the later tillers. Additionally, the author has observed mottling and speckling in certain genotypes that are uninfected with bunt (Patterson, *unpublished data*) suggesting that this may be an environmental response. More resistant cultivars may show no reduction in tiller height or stand, with heads showing only a portion of bunted kernels. They may even have only partially bunted kernels. This wide range of disease phenotypes would be inconsistent with a solely gene-for-gene system in which

resistance genes were able to fully exclude the fungus and the hosts lacking any resistance genes would be fully susceptible.

A certain degree of quantitative resistance and field resistance to common bunt has been suggested by Gaines (1920), Pope and Dewey (1975), Gaudet and Puchalski (1989), Eibel et al. (2005), Fofana et al. (2008), Bokore et al. (2019), Muellner et al. (2020a), Muellner et al. (2020b), and Steffan et al. (2021). Quantitative disease resistance is the reduction (rather than total prevention) of disease and is a quantitative trait. Generally, quantitative disease resistance is the product of many smaller resistance-adjacent genes that can be influenced by the environment or other minor genes and is race non-specific (St. Clair 2010). Quantitative resistance to common bunt in wheat is supported, in theory, by segregation patterns showing partial resistance in certain progenies (a gradient of infection within a population or individual plant rather than total or absent infection similar to stripe rust quantitative resistance (inexplicable by Mendelian genetics), the wide range of phenotypic resistance patterns implying "degrees of increasing resistance," the interpretation of "threshold effects" (developmentally-implicated differences in intraplant susceptibility) (Pope and Dewey 1975), and the identification of major and minor resistance quantitative trait loci (QTL) (Fofana et al. 2008; Bokore et al. 2019; Muellner et al. 2020b, 2020a). Pope and Dewey (1975) observed a "continuous array of resistant phenotypes from high to low percent smut" that they attributed to combinations of genes *Bt1, 3, 4, 7, 9,* and *10* in their study population.

In Gaudet and Puchalski's 1989 study of bunt resistance sources in Canadian spring wheat and triticale, they observed field resistance of their test plants to common bunt. Gaudet and Puchalski (1989) observed hard red spring wheat varieties that were broadly resistant in the field but susceptible to every race tested in controlled experiments. The authors discussed the possibility of a race nonspecific field resistance and the possibility of other resistance genes that were at that time undetectable. They, much like Pope and Dewey, were troubled by the categorization of cultivars averaging between 10-20% infection as either resistant or susceptible.

In 1930, Woolman proposed a certain level of innate immunity even in the most susceptible plants. In studying the progression of the infection in susceptible plants, he

found that the infections reaching the developing tissues were those of four or less individual infections (infections from a single germination event at a single point of entry), although no fewer than 100 points of infection were recorded. Concluding that most infections are stopped in the early stages, he suggested that there were inhibiting factor(s) present in the plant that generally prevent the development of bunt in the wheat plant.

A later study suggested that resistance in a wheat variety thought to be fully resistant ('Baart 38') was confined to the suppression of spore production. Mycelium (ostensibly of common bunt) was found throughout the plant at all stages of growth and bunt teliospores were found in healthy kernels of the resistant variety. This study did not report the genetic basis of resistance in 'Baart 38', though it would be interesting to know which gene(s) were associated with this response (Griffith et al. 1955, 1953). The study, also, was performed with a fungal-specific stain but their methods may have allowed for the observation of endophytes as well as pathogens.

To add to the body of evidence for incomplete resistance and susceptibility, Pope and Dewey (1975) shared observations from both their breeding populations of wheat. In general, their data could not be explained by major resistance genes alone and no one resistance gene provided complete resistance in the host, giving rise to the idea that major resistance genes are accompanied by minor or resistance-associated genes. One of Pope's cultivar lineages, derived from a cross of 'ID5011'/'ID5006', showed a segregation pattern consistent with the presence of four or more genes when challenged with dwarf bunt. Other families produced one of 5 disease phenotypes: 5, 10, 20-30, 30-40, or 50-60% bunted heads. These families, in addition, produced at minimum "three wide and three narrow segregation patterns" that were interpreted as the representation of various combinations of resistance genes. In Dewey's populations, crosses between a cultivar possessing *Bt1*, *Bt3*, and *Bt4* ('Delmar') and a cultivar possessing *Bt9* and *Bt10* ('PI178383') produced offspring in which *Bt9* and *Bt10* segregated out in the population between very susceptible progeny and very resistant progeny. On their own, *Bt9* and *Bt10* permitted 20-25% bunting under severe disease pressure, but this was drastically reduced with the addition of unknown genes and drastically heightened with the removal of either gene. Crosses between a cultivar

possessing *Bt1* and *Bt4* ('Columbia') with 'Delmar' produced offspring in two groups; a group in which *Bt1* and *Bt4* produced resistance and another which were susceptible to the same mixture of common bunt. What, then, was the function of *Bt3* on its own? The data suggested, as has since been suggested for other named resistance genes (Muellner et al. 2020b; Chen et al. 2016), that *Bt3* is actually a complex of individually weaker resistance genes. In particular, a line released from that cross ('Bridger CI 14580') showed slightly more susceptibility to the common bunt races and slightly less to the dwarf bunt, indicating that although it had the same major resistance genes as its parents, it likely had a different set of minor resistance-associated genes. However, as noted by St. Clair (2010), the continuous distribution of disease phenotypes through a population isn't necessarily explained only by multiple loci. Other explanations such as "a gene controlling a trait with low heritability (proportion of genotypic to phenotypic variance)" or "high environmental influence on trait expression" could explain such a distribution as well as a multi-gene scenario with high heritability. St. Clair (2010) notes that segregation data must be supported by genetic analyses, such as the identification of quantitative trait loci.

In 2008 Fofana et al., working in Canada, identified three quantitative trait loci implicated with field/non-race specific resistance in Canadian spring wheat. Quantitative trait loci refer to "genomic region[s] containing one or more genes that exhibit a statistically significant association between marker polymorphisms and quantitative trait variation" (St. Clair 2010) and they contribute to quantitative disease resistance, at which point they may be referred to as quantitative resistance loci (QTL). When QTL are "mapped," they are identified and located on the chromosome using computational software by the frequency with which they are related to a specific phenotype of interest. After noting that some elite germplasm, notably 'Yaroslav Emmer' and 'Marquis' have been used as a source of disease resistance without the specifically reported presence of *Bt* genes, they investigated a doubled haploid population from another variety of that lineage finding the aforementioned QTL. They interpreted the continuous distribution of disease reactions among the doubled haploid population, the ability of the QTL to explain disease phenotypic variation, and the location of the QTL relative to previously mapped genes as evidence of some sort of

minor-gene resistance to common bunt. Specifically, the disease distribution was interpreted as a suggestion of the oligogenic quantitative inheritance of field resistance. In addition to their description of the three QTL they did discover, they posited that more minor QTL were likely present but the moderate heritability of resistance, combined with trial-to-trial phenotypic variability and the limitations of the technology at the time placed the minor QTL below their ability of detection (Fofana et al. 2008). Minor QTL are those QTL explaining less than 20% of phenotypic variation, whereas major QTL explain more (St. Clair 2010). Both, however, are considered minor resistance genes.

The presence of minor resistance genes was supported by work in Canada in 2019. The researchers noted that a Canadian Western Red Spring wheat variety, 'Lillian', had a moderate degree of resistance to the Canadian bunt population and so set out to map QTL associated with that level of moderate resistance. Using a doubled-haploid (DH) population from a crossing of 'Lillian' and 'Vesper,' they were able to identify two stable common bunt resistance QTL on chromosomes 5A and 7A and three less stable QTL (Bokore et al. 2019). The phenotypes of disease incidence showed a "skewed continuous distribution toward resistance," indicating that multiple minor resistance genes with cumulative effects were responsible for the plant's ability or inability to exclude the pathogen. They divided their DH lines into groups by the combinations of their 5 QTL present in those lines and found that their lines with none of the identified QTL were still less diseased than their susceptible check, which they attributed to either QTL existing but not statistically identified or interactions between the genotype and environment. Interestingly, two of the three less stable common bunt resistance QTL were inherited from the moderately susceptible parent 'Vesper' – suggesting that even the moderately susceptible cultivar possessed unique (although incomplete) resistance genes that would be completely separate from the major *Bt* genes currently identified. They noted that the multigenic resistance in 'Lillian' was as effective as the monogenic resistance in 'AC Cadillac.' Even further, Bokore et al. (2019) found evidence of epistatic interactions between a less stable QTL and a stable QTL that together increased resistance to common bunt.

Yet another study conducted internationally in 2020 investigated the location of *Bt12* using a mapping population of 176 recombinant inbred lines (RIL). In observing the incidence of common and dwarf bunt in their trials, they found evidence of quantitative variation in all the trials they conducted. They reported a pattern of a "positively skewed continuous distribution, with a large proportion of lines in the low or not infected groups" (Muellner et al. 2020b). Although there was high disease pressure in their trials, the variation in resistance and high proportion of RILs with a high yet incomplete resistance to common and dwarf bunts is evidence for other minor resistance factors in the resistant parent segregating out in the mapping population (Muellner et al. 2020b). Their study was particularly interesting as a testament to the commonality of wheat resistance to CB and DB simultaneously, with the same QTL highly relevant to both CB and DB resistance. Muellner's team published another study on common and dwarf bunt resistance in bread wheat in that same year, reporting that in RIL populations QTL for common and dwarf bunt resistance could be mapped to chromosomes 1AL, 1BS, 7AL, and 7DS (Muellner et al. 2020a). In this study, the researchers also provided support for quantitative resistance. Quantitative variation, they reported, "was evident in all trials, which generally followed a positively skewed continuous distribution with more than 50% of lines showing low (<10% bunt incidence) or no infection." Although their other work clearly demonstrated the commonality in wheat resistance to common bunt and dwarf bunt, this study was able to detect different QTL for common and dwarf bunt resistance in their RIL population. Common bunt resistance was regulated by 2 major QTL and 1 moderate effect QTL, while dwarf bunt resistance was regulated by three QTL only one of which was in common with common bunt. In discussing the heritability of resistance, Muellner et al. (2020a) write that "quantitatively inherited resistance is complementary to race-specific *Bt* genes."

During host resistance interactions, many defense-related genes may be up-regulated or down-regulated (Matanguihan et al. 2011). In general, plants produce a series of phytohormones in response to recognition of invading pathogens, including fungal pathogens. These phytohormones regulate the plant defense response and lead the plant in accumulating pathogenesis-related (PR) proteins. The genes coding for the

PR proteins increase their expression rapidly following the detection of infection, and one or more of 17 PR protein families may be induced (Ali et al. 2018; van Loon et al. 2006). These proteins can induce the release of biochemicals that activate defense-related signal cascades or directly damage the pathogen. A 2005 study of *Bt10* and *T. laevis* race T-1 observed 168 differentially up-regulated and 25 down-regulated genes, most of which had homology to genes known to function in cellular metabolism and development, stress response, transcription and signal processes, two putative resistance genes, and a transcription factor (Lu et al. 2005a). Other studies have confirmed higher expression of several candidate genes (a lipase, two non-specific lipid transfer proteins, and wheat pathogenesis-related proteins) in resistant reactions over susceptible reactions (Lu et al. 2005b).

Work in 2020 built on a general understanding of plant defense responses to target and evaluate the expression of pathogenesis-related proteins. The team observed 5 pathogenicity regulators and 5 PR genes expressed consistently more in a resistant cultivar than a susceptible cultivar, and witnessed their expression increasing in contrast to the susceptible cultivar over the course of a week. This higher expression was positively associated with successful resistance to dwarf bunt (Muhae-Ud-Din et al. 2020).

A study published in March 2021 reported that several defense-related genes were expressed more in response to *T. controversa* infection in resistant wheat cultivar 'Yinong 18' and slightly more in a moderately resistant cultivar 'Pin 9928' than in the susceptible cultivar 'Dongxuan 3.' They investigated defensin, *TaPR-2*, and *TaPR-10*, all of which have been classified as defense response genes. They posit that these genes, therefore may possibly be responsible for regulating resistance in resistant to moderately resistant cultivars (Chen et al. 2021).

An October 2021 study reported that, in wheat spikes infected with the nearly conspecific *T. controversa*, defense-associated genes were more highly expressed. These genes included well-established defense genes; "PR-related genes, WRKY transcription factors, and mitogen-activated protein kinase genes" (Ren et al. 2021a).

Published research shows *Bt1*, *Bt3*, *Bt4*, *Bt5*, *Bt6*, *Bt7*, *Bt8*, *Bt9* and *Bt10* have been studied in varying degrees of detail. Although mapping studies performed before

the advent of SNP and GBS mapping techniques are no longer considered fully adequate, the following studies formed a basis for further study of resistance genetics in the common/dwarf bunt and wheat pathosystems. *Bt1* was mapped to chromosome 2B (McIntosh et al. 2003; Sears et al. 1960). Three genes (*Bt4*, *Bt5*, and *Bt6*) were mapped to chromosome 1B (McIntosh et al. 2003; Schmidt et al. 1969). *Bt7* was mapped to chromosome 2D (McIntosh et al. 2003). *Bt10* has been mapped to the short arm of chromosome 6D (Menzies et al. 2006). Since these publications, different mapping tools have been developed and become available.

Further mapping efforts are accompanied by the development of QTL and SNP markers thought to be associated with genes of interest, for example *Bt9* is located on chromosome 6DL (Steffan et al. 2017), and has been associated with the QTL *Q.DB.ui-6DL* (Wang et al. 2019). A TG25K array has been used to confirm the locations of *Bt1* on chromosome 2B, *Bt5* on chromosome 1B, and *Bt9* on chromosome 6D (Christensen and Borgen 2021b). Christensen and Borgen's 2021 work confirms the work of Steffan et al. (2017) in mapping *Bt10* to chromosome 6D. Interestingly, 6D seems to be a chromosome with a concentration of *Bt* genes. Along with *Bt9* and *Bt10*, it is hypothesized that *Bt8* may be tightly linked to *Bt10* again on chromosome 6D (Christensen and Borgen 2021a). Efforts to map *Bt11* are underway as well. A 25k SNP platform, which utilizes many oligo probes in a specific arrangement to detect SNPs between the genomic DNA of different samples (Baćanovic-Šišić et al. 2021b), is being utilized to genotype mapping populations (Ehn et al. 2021). The TG25K array has also suggested that *Bt12* may be located on chromosome 7D (Christensen and Borgen 2021b), which complements results from 2020 locating *Bt12* on chromosome 7DS (Muellner et al. 2020b). Other SNPs have been identified, but not yet associated with a specific resistance gene. For example, a 2018 study associated 15 SNPs with common bunt resistance, detecting them on chromosomes 1B, 2A, 2B, 3D, 4A, 7A, and 7B (Bhatta et al. 2018). In the same year, 123 SNPs from a diversity panel of 330 winter wheat selections were detected on 14 chromosomes (Mourad et al. 2018). In 2021, another group identified 14 SNPs on chromosome 1A, 12 on 7A, and 11 on 2B (Baćanović-Šišić et al. 2021).

In recent years, the study of QTL involved in wheat resistance to bunt has identified over 25 QTL. These QTL are believed to be an essential part of non-race specific bunt resistance (Muellner et al. 2020b). So far, QTL have been identified on several chromosomes. In 2008, QTL were described on 1B and 7A (Fofana et al. 2008). In 2009, on 1B again (Wang et al. 2009). In 2012, on 1B again and 5B (Dumalasová et al. 2012). In 2013, chromosome 7B was linked to resistance (Knox et al. 2013). In 2016, 2 studies were published. One found QTL on 1B again, further confirming previous reports, and 4B, 4D, 5B, and 7DL (Singh et al. 2016). The other 2016 study identified a major QTL for dwarf bunt resistance on chromosome 7D's short arm (Chen et al. 2016). An additional study weas published in 2017, locating QTL on 1B again and 3A (Zou et al. 2017). In 2019, QTL were found on 1D, 2A, 3D, 5A, and 7A (Bokore et al. 2019). As the number of studies progressed, they continued to both confirm prior findings and report more novel QTL. In 2021, QTL were found at 2B and again at 7A (Steffan et al. 2021). In 2020, Muellner et al. used a mapping population derived from a *Bt12* differential ('PI119333') and a susceptible variety ('Rainer') to identify both a major effect QTL and a minor effect QTL on chromosome 7D and 7DS, respectively. Their major effect QTL, *QBt.ifa-7DS*, is likely highly related to *Bt12*. Given that, to the author's knowledge as of August 2021, the series of 6 genes (*Bt2*, *Bt3*, *Bt13*, *Bt14*, *Bt15*, and *Btp*) have not been successfully mapped, it is possible that the SNPs and QTL associated with the 6 chromosomes 1A, 2A, 3D, 4A, 7A, and 7B may each correspond to an unmapped gene.

In addition to the discussion of quantitative resistance and regulation of defense genes, the concept of a gene-for-gene response as the sole arbiter of disease outcome has been challenged in this pathosystem, other pathosystems, and in general. The concept of phytoimmunity or nonhost resistance, in which most plants are able to resist most pathogens, illustrates a complex network of plant responses to a number of pathogen stimuli. While the gene-for-gene concept is still a useful understanding of the plant resistance response, the addition of the modern concept of innate immunity posits that "plant immunity is the result of a multi-layer innate immune system having various structures and mechanisms of both specific and non-specific immunity" (Shafikova and Omelichkina 2020). Host resistance may then exist in a "gray zone" or continuum between qualitative gene-for-gene systems and quantitative disease resistance (St. Clair

2010). In the past decade, defense responses have been understood to be triggered by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). PAMPs are molecular structures or patterns that are intrinsic to the pathogen, but foreign to the host (Jones and Dangl 2006). The focus on PAMPs and DAMPs is understood in the context of sub-genetic protein-protein interactions that are also under continued study. While Flor (1971) usefully distilled a complex gradient system of categorizing resistance into a binary, it is more accepted now that a resistance gradient is a likely outcome of protein-effector interactions in which no protein has absolute specificity, no "signaling cascade" is cleanly arranged, networks of interactions surrounding "hubs" abound, and intermediate steps may not exist (Gassmann and Bhattacharjee 2012). While genetics has been useful in pinpointing the architecture of resistance, it has been thus far unable to determine the molecular interactions that protect plants (Gassmann and Bhattacharjee 2012).

Bt10 is a major gene used in resistance breeding programs, resistant to 35 of the 40 known bunt races as of 2011 (Matanguihan et al. 2011). *Bt10* is largely considered one of the most effective resistance genes globally (Madenova et al. 2021), and it holds particular importance in Canada where it confers resistance to all races of common bunt present in the country (Dumalasová and Bartoš 2016; Fofana et al. 2008). *Bt10* is inherited in a partially dominant fashion (Laroche et al. 2000). In *Bt10* resistance, infection does occur, but the incompatible reaction restricts the hyphae (at least of race T-1) which are prevented from spreading. This may be related to a buildup of callose, a cell wall reinforcing material, around the infection site which can provide a physical barrier to the spread of the bunt hyphae (Muhae-Ud-Din et al. 2020; Gaudet et al. 2007; Kudlicka and Brown, Jr. 1997). Likely enabled by other unknown plant-produced fungistatic/fungitoxic antimicrobial factors that may inhibit pathogen growth, microscopic callose droplets begin appearing/forming in infection-adjacent cells 8-10 days after seeding, corresponding with a 4-6 day period after germination of teliospores. These droplets eventually aggregate and tightly press to the cell wall within 2 cells of the infection site, forming successive layers around the hyphae. Once covered in callose, infection hyphae become dark, the cell wall degrades, and the cytoplasm dissipates (Gaudet et al. 2007).

It's possible that the resistance response of genes such as *Bt10* are involved in the recognition of PAMPs that result in the swift blow of PAMP-triggered immunity (PTI) to the invading common bunt. PTI involves the activation of basic, non-specific plant responses, of which the accumulation of reactive oxygen species (ROS) and nitric oxide (NO), the synthesis of phytoalexins, cell wall lignification, and deposition of callose are prevalent and seemingly conserved components (Shafikova and Omelichkina 2020; Hemetsberger et al. 2012). For common bunt to suppress PTI indicates that the action of avirulence (*Avr*) genes must be to either avoid host recognition or suppress the host defense response. For resistance to occur, the action of wheat resistance genes must be to either recognize PAMPs (such as chitin) or effectors. However, these resistance genes are unlikely to form an entire immune response on the basis of one gene. When Gaudet et al. (2007) elucidated the role of callose in the resistance of wheat to common bunt, it's possible that they were looking at a highly conserved response in the wheat plant. The rapid deposition of callose could potentially be due to a pattern-recognition receptor (PRR), given both that infection was cut off swiftly and there was no hypersensitive response (HR). A lack of HR, which is a characteristic of many effector-triggered immunity (ETI) responses, was observed and could indicate that the PTI is not overcome by that race of bunt. Indeed, wheat and other plants are known to deposit callose in response to many other pathogens**.** It is, however, important to note that callose deposition in any pathosystem depends on the environmental conditions and specifics of the pathogen challenging the host (Luna et al. 2011). While it is common, it also depends on several pathways in different situations and in response to any of the multiple PAMPs associated with any one species (Luna et al. 2011) and may be regulated in part by genetics.

In addition to the idea that *Bt10* may be a gene associated with PAMP recognition and PTI, there is room to believe that other *Bt* genes could be involved in varying portions of Jones and Dangl's (2006) "zig-zag model," responding to either primary or secondary effectors. If this is the case, differing levels of infection may occur based on the action of the gene. Quantitative resistance loci, corresponding to quantitative resistance genes, are hypothesized to use any of a number of biological pathways to confer resistance; the genes may regulate morphology or developmental

traits, they may be allelic mutations in basal defense genes, they may affect or induce chemical warfare compounds, or be involved in defense signal transduction (St. Clair 2010). With the variety of modes that minor resistance genes may take; it is extremely likely that different major genes may play different roles as well.

According to speculation by Gaudet et al. (2007), resistance is likely not a systemic response to the pathogen. They observed an immediate walling off of the invading fungus by callose deposition in a *Bt10* differential. They argue that due to the aggressive nature of the fungus once it reaches the growing point it is unlikely that "defense responses initiated in an incompatible interaction are maintained much beyond the point of penetration" or that a systemic resistance response is involved in resistance to this type of pathogen. Otherwise, the pathogen could never maintain the growth rate necessary to attain even low levels of infection and proliferate in the developing spike, if it was subjected to a continuous defense response." In response to other findings of low levels of infection in incompatible reactions, the team points to known environmental moderation of resistance and susceptibility, citing Gaudet & Puchalski's 1995 paper on the relationship between temperature and bunt resistance. The combination of genes *Bt1*, *Bt3,* and *Bt4* was studied in a wheat/*T. controversa* pathosystem. The cultivar 'Nugaines,' which possessed all three genes, demonstrated a temperature-sensitive resistance that did not exclude *T. controversa* from colonization of the 1st and 2nd leaf primordia, but did exclude it from the growing point (Fernández et al. 1978). Madenova et al.'s 2021 screening of a plethora of cultivars over three years found varieties that were resistant in certain years and susceptible in others. Of the 61 varieties tested, only 7 displayed high resistance across all three years. Although they don't specifically interpret this as environmental variation, it seems highly likely to the author that this is further evidence of the phenomenon. In conjunction with the observations of Woolman (1930) and others, this signifies that resistance may be achieved in an environmentally-dependent or R-gene-dependent manner. Resistance effectivity can be decreased if the plant encounters cold temperatures (approximately 10°C) during early development (Griffith et al. 1955; Smith 1932). Certain cultivars express increased disease growth when exposed to higher temperatures (Fernandez et al. 1978). In general, Fernandez et al. (1978) posited that there are two forms of

resistance to dwarf and common bunt: temperature-dependent and environmentally-independent. Goates, in his 1996 review of common and dwarf bunt, indicates that some winter/spring facultative cultivars may be susceptible in fall plantings and resistant in spring plantings, which has been interpreted as evidence for the existence of environmentally-sensitive resistance genes. Bokore et al. (2019) explicitly cited environmental variation as a moderating effect on minor resistance genes. Earlier work has determined that bunt infection is likely a product of many factors, with photoperiod among them. Bunt incidence was significantly increased when plants experienced a 14.5-16 hour day length (Zscheile 1966). It was hypothesized that these observed environmental effects are compounded in research projects by the likelihood of changes in the fungus in culture during storage (Thomas 1991).

Genetic resistance is, evolutionarily, a temporary status. Host resistance, especially where moderated by a limited number of resistance genes, provides only cultivar-specific resistance that in turn provides intense selection pressure for pathogen evolution when varieties are grown in monocrops (Gill et al. 2015; Dangl et al. 2013) as is standard for wheat. Due to the constant evolution of the pathogen, new resistant varieties and understanding of resistance are always necessary (Goates 1996).

Diagnostic Tools

The earliest methods of detection involved looking for intensely verdant young ovaries that were unlike the healthy young, white ovaries (Fischer and Holton 1957). This method was improved by the staining of tissue (Swinburne 1963; Hansen 1958). Swinburne (1963) used both lactophenol cotton blue and Johansen's quadruple stain to differentiate all cell types within the samples. In 1987, a method previously used for *Ustilago nuda* involving autoclave-based tissue clearing and staining with 1% aqueous trypan blue was used successfully to correlate the presence of hyphae in seedlings to disease incidence at harvest (Kollmorgen and Ballinger 1987). 1% trypan blue, in conjunction with the application of either potassium hydroxide (KOH) or heat damage and formaldehyde, is able to stain intercellular fungal cell walls differentially from plant cell walls (Wilkes et al. 2020). In 2006, Kochanová et al. published a summary of laboratory diagnostics for common bunt (and dwarf bunt) at the juvenile stages. The

team reviewed the staining of mycelium with 1% trypan blue compared to PCR and dot blot hybridization. Both PCR and dot blot hybridization were found to be specific to the common and dwarf bunt fungi group, with their presence confirmed by the trypan blue staining (Kochanová et al. 2006).

PCR-based diagnostic assays are powerful tools to detect and potentially quantify the presence of specific pathogens in a host due to their sensitivity, reliability, reduced timeframes over culture-based methods, and detection of minute quantities of DNA (McNeil et al. 2004; McCartney et al. 2003). PCR-based methods are useful for studying causal organisms before they develop disease, which is useful in screening germplasm for pathogen resistance (McCartney et al. 2003). In other host-pathogen systems, the amount of fungal DNA has been correlated to the extent of resistance/severity of infection including fusarium head blight in wheat (Horevaj et al. 2011; Burlakoti et al. 2007), wheat tan spot and glume blotch (Abdullah et al. 2018a), wheat eyespot (Meyer et al. 2011), loose smut of barley and wheat (Wunderle et al. 2012), *Aphanomyces euteiches* and *Phytophthora medicaginis* in alfalfa (Vandemark and Barker 2003; Vandemark et al. 2002). Similar correlations have been published for common bunt of wheat (Orgeur 2021; Zhang et al. 2012b; Josefsen and Christiansen 2002). Thus far, PCR-based methods have been successfully used to detect common bunt at the first node stage (Josefsen and Christiansen 2002), second to third leaf stages (Orgeur 2021), second and fourth leaf stage (Eibel et al. 2005), in apical meristems (Zouhar et al. 2010), and in washes of wheat grain by real-time PCR (Forster et al. 2021; Zgraja et al. 2016; McNeil et al. 2004).

In 2002, Josefsen and Christiansen used nested PCR, amplifying the ITS2 region first and subsequently amplifying a target region of that fragment to detect bunt hyphae in seedlings. In 2004, Kochanová et al. published a PCR method to detect *T. caries* as well as *T. controversa*, noting that the similarities between the genetic sequences of the two fungi were difficult to overcome in designing primers for one or the other specifically. However, the assays must be specific only to the target pathogen, sufficiently sensitive to small concentrations of pathogen DNA within host samples, and tolerant of variations in total DNA quantity in the sample (Admassu-Yimer et al. 2019) in order to distinguish between dwarf and common bunt. Additionally, traditional PCR

methods are incapable of quantifying the amount of target DNA initially present in the sample (McCartney et al. 2003).

Eibel et al. (2005) developed a PCR test to compare it to ELISA-based methods and found that their ELISA method was incapable of detecting spores, showing that qPCR could be used for soil and seed sampling. However, this also indicates that ELISA is more suited to diagnosing and quantifying common bunt infection in the developing plant given it would not account for spores that had not germinated yet on the surface of the plant. ELISA, though, relies on the availability of monoclonal antibodies while primers for PCR and qPCR are easily purchased commercially. Finally, ELISA is generally less sensitive in diagnostic capacities than PCR-based methods (Wunderle et al. 2012).

Real-time PCR (qPCR), a variation of traditional PCR developed in the mid-1990s, uses one of two popular potential assays with different modes of action to quantitatively measure DNA quantities by the fluorescence of an associated dye. While both assays rely on integrated cyclers/fluorimeters to measure the accumulation of DNA (McCartney et al. 2003), one assay is sequence-specific (TaqMan) and the other sequence non-sequence-specific (SYBR Green). SYBR Green dyes may intercalate with any double-stranded DNA (dsDNA) (Heid et al. 1996; Schena et al. 2004; Admassu-Yimer et al. 2019). This may result in false positives, making TaqMan chemistry the superior choice for fungal DNA quantification (Admassu-Yimer et al. 2019; McNeil et al. 2004). TaqMan chemistry relies on the action of fluorophores that are in close proximity to quenching dyes. Both dyes are bound to a nucleotide sequence complementary to a region of the PCR product, and the closeness of the quenching dye to the fluorophore prevents the fluorescence emission of the fluorophore by Förster resonance energy transfer (FRET) (McCartney et al. 2003). When the fluorophore + quenching dye + nucleotide sequence complex binds to the DNA target, the quenching dye is released by the DNA polymerase's 5' endonuclease activity and the newly distanced fluorophore emits fluorescent light proportional to the present quantity of PCR product that is then recorded by the qPCR thermocycler's fluorimeter. In either qPCR method, a threshold is set at the mean standard deviation of ΔRn (the difference between the reacted and unreacted sample) early cycles and a Ct value (previously and

additionally referred to as a "Cq" value) is calculated to describe when the measured fluorescence in a well surpasses this threshold - in other words "the cycle number at which a statistically significant increase in the ΔRn … is first detected" (McNeil et al. 2004). Using the theory that more DNA in a well will correspond to fewer cycle numbers to cross the threshold, the Ct value can be compared to a standard curve to quantify the amount of DNA in a well or can be used to determine a relative amount of DNA when compared to another fluorophore in the same well.

McNeil et al. (2004) write that their TaqMan qPCR has several advantages over PCR or microscopic methods - it is less labor-intensive, has a higher throughput, requires less training than morphological identification, and can detect very low levels of bunt infection. In 2010, Zouhar and his team published a SYBR Green I qPCR assay for quantifying common bunt in apical meristems. Their assay was able to detect mycelial DNA amounts as low as 0.22 ng. While many qPCR assays for quantification of common and dwarf bunt pathogens have been published (Zouhar et al. 2010; McNeil et al. 2004), the lack of systematic experimental conditions and specific validation of references has led to irreproducible results with questionable reliability (Scholtz and Visser 2013; Bustin et al. 2009). Adding a host target sequence to the TaqMan qPCR assay has been shown to normalize DNA quantification results against variance in the initial quantity of DNA (Admassu-Yimer et al. 2019; Acevedo et al. 2010). McNeil et al. (2004) published a qPCR assay for *T.* common and dwarf bunts sensitive to 1 spore/seed (McNeil et al. 2004).

Two problems are important, though, when considering the use of PCR-based diagnostic methods. First, as discussed previously, is the evaluation of the isolates used in the development of the assay. If insufficient isolates were used, the assay may not be applicable at the scale intended or necessary (Bao 2010). Another problem with existing PCR-based diagnostic tools is that many of the methods that had been published as of 2019 had focused on single-locus differences between different species of *Tilletia* (Nguyen et al. 2019). Nguyen et al. (2019), therefore, developed an *in silico*-tested primer and probe catalog for differentiation of the quarantine dwarf bunt from the non-quarantine common bunt fungi based on their own assembly of the three genomes. Due to these problems, potentially alongside the issue of a lack of PCR-based

differentiation between common and dwarf bunt, as of 2021 none of the previously published PCR-based assays are in use as standard practice (Forster et al. 2021). Identification and quantification of teliospores in seed lots is still heavily reliant on filtration of the seed lot in accordance with the International Seed Testing Association guidelines, followed by microscopic examination by well-trained individuals (Forster et al. 2021). While Forster et al. (2021) put forward a new probe-based qPCR method to differentiate between the two diseases' causal agents based on a large number of genomes and isolates, it remains to be seen whether this promising assay will be widely adopted.

In 2018, a loop-mediated isothermal amplification (LAMP) method was published for identification of *Tilletia* spp. in seed lots. LAMP assays generally use four to six primers to bind six to eight target DNA regions - amplifying with high specificity the target DNA in a short time without the need for thermal cycling. Products may then be analyzed by gel electrophoresis, direct staining, or continuously monitored by real-time detection. While the assay is able to differentiate *T.* common and dwarf bunt species from other common fungi, it is not able to differentiate between *T. laevis, T. caries* and *T. controversa* (Pieczul et al. 2018).

Gaps in the Literature

According to Matanguihan et al. (2011), at of the end of the 2000s, research on resistance was "echoing" the work that had been done in the early 20th century. Studies on bunt incidence, cataloguing new races of the *T.* species, resistance screening, looking at modes of resistance inheritance and trying to identify new sources of resistance were at the forefront of research efforts. Using newer technology, research also turned to identification and mapping of resistance genes and associated QTL (Muellner et al. 2020b; Wang et al. 2009; Fofana et al. 2008; Menzies et al. 2006). While this has improved our understanding of the genetic basis of resistance and our knowledge of the natural incidence of bunt, there are gaps in our understanding of the way the fungus interacts with its host and the ways in which resistance is effectively accomplished. While marker-assisted selection (MAS) is a highly useful tool for pathosystems

displaying complex inheritance of resistance (St. Clair 2010), these gaps impact our ability to breed new resistant varieties, given the lack of support for effective MAS (Muellner et al. 2020b).

While several studies, as discussed, have investigated various plant tissues with PCR-based methods, no studies have been able to quantify specifically common bunt in developing tissues, or attempted to correlate fungal biomass with visualization and disease incidence. To the author's knowledge, only one such study has been published for dwarf bunt (Chen et al. 2021). PCR-based methods have been used to create diagnostic assays, but they have largely focused on destructively sampled single tissue in various growth stages and thus have not characterized the growth of the fungus throughout the entire life cycle of the plant. For example, though Josefsen and Christiansen's (2002) diagnostic assay was well characterized - they looked only at the wheat inflorescences cut from plants at the first node stage. While the works of Hansen (1958) and Swinburne (1963) are thorough anatomical investigations of a susceptible reaction, they did not compare incompatible reactions for the races or resistance of the biological material used. Nor did later, more thorough and modern studies such as that of Ren et al. (2021b).

While Gaudet et al.'s 2007 study seems to be the first to investigate and visualize the action of a particular resistance gene, similar studies of other genes do not seem to have followed. There is room to postulate that, given the knowledge of separate resistance gene responses to different environmental factors and various observations of the growth of the pathogen in otherwise asymptomatic plants, different resistance genes would exclude the pathogen from developing disease in different ways. In other pathosystems, it is noted that race-specificity is a feature of quantitative disease resistance that indicates that not all host genotypes would respond to different races in the same manner (St. Clair 2010).

Gaudet et al. (2007) also note the areas that need further exploration, stating that the use of multiple races to challenge a resistance gene may be necessary to the study of that gene's function. It is possible that the same resistance gene may be altered in its effects by the presence of different pathogen races, which may account for the

compatibilities and incompatibilities of different host/pathogen genotype combinations.

In the future, once individual genes are better understood, the interactions of pyramiding genes might be worth studying. If it is true that different resistance genes work in different ways and trigger separate defense cascades, it would then be more than likely that these cascades would either be altered or enhanced with gene pyramiding.

Additionally, there is room to improve our understanding of resistance genotypes in the most successful resistant varieties currently available - although Fernandez et al. (1978) postulate that those interactions between bunt and resistance genes are unlikely to be highly variable due to the commonalities in wheat development across cultivars and time/place of infection and the breadth of gene-for-gene reactions and additional unknown factors.

Additional work could be undertaken to investigate the possibilities of innate or systemic resistance within wheat differentials, as well as the possibility of quantitative resistance. It is hypothesized by Gaudet et al. (2007) that resistance is not a systemic response, but rather a local response at the time of penetration. If this were not the case, they argue, it would be impossible for the fungus to ever spread throughout the plant in competition with a systemic resistance reaction to the point of infecting the ovary and forming bunt balls at the low disease incidence of up to the 8% that is observed. However, general host resistance is still possible and, as discussed, quantitative resistance has been postulated by several authors. While systemic responses may be unlikely, there are gaps in our understanding of fungal-host resistance systems, especially in the lesser studied common bunt.

An integral part of any pathosystem is the interaction between pathogen *Avr* genes and gene products with host machinery. As of my literature review in 2022, no papers identifying common or dwarf bunt avirulence genes have been published. For the closely related Karnal bunt (*T. indica*), another devastating wheat disease in the same genus not currently controlled, the whole genome was sequenced in 2019 (Gurjar et al. 2019). In doing so the researchers were able to identify a number of pathogenesis-related genes, which could then be used to understand the mechanisms of Karnal bunt

pathogenesis, the intricacies of the fungal life cycle, and teliospore survival. This knowledge will facilitate faster development of control measures and enhanced management and breeding strategies against Karnal bunt. Although the whole genome of common and dwarf bunt has been sequenced (Nguyen et al. 2019), this information was used to identify species-specific genetic markers for identification of the pathogens. In the future, these genomes could be used in the way the Karnal bunt genome is in use to identify pathogenesis-related genes and elucidate the genetic underpinnings and mechanisms of common and dwarf bunt virulence.

Conclusion

Common bunt was a scourge on wheat prior to the 1950s, with significant impacts on crop production going as far back as recorded history. The aggressive colonization of the host plant, ability to proliferate in either a seed- or soil-borne manner, adaptability, and wide geographic range made it a terror. While many effective fungicide seed treatments have been produced since the 1950s, societal ideas about what qualifies a wholesome food are changing and organic markets have excluded the use of such chemical seed treatment fungicides. As organic acreage grows, the threat of major common bunt outbreaks likewise grows.

Dwarf bunt, while closely related to common bunt, poses its own unique threat to wheat production. Though they share similar morphologies, genetics, and life cycles, slight differences in environmental requirements slow the spread and delimit the yearly impact of dwarf bunt. These differences made chemical control of dwarf bunt, which is a soilborne and not a seedborne pathogen, more difficult to develop and even now there is only one seed-treatment fungicide (difenoconazole) on the market for dwarf bunt prevention. However, as a quarantine pathogen commonly confused with common bunt, dwarf bunt must be all the more carefully excluded from internationally-bound grain. Given that the common bunt and dwarf bunt (*T. controversa* J.G. Kühn) fungi are similar enough to be potentially considered conspecific, either common bunt species may be used to study the infection process of both bunt diseases. Due to the shared nature of effective resistance genes to common and dwarf bunt, investigations into

resistance to common bunt are largely applicable to dwarf bunt and thus serve a dual purpose.

Developing effective management strategies utilizing resistance and non-chemical control measures rely on an in-depth understanding of the fungal pathogens. The detection and quantification of *T. caries* and *T. laevis* throughout the host life cycle is advantageous for resistance breeding, as it may inform our understanding of resistance. Resistant cultivars are the most feasible option in terms of combating common bunt in a way that can be most easily distributed equitably on a global scale. Despite high breeding costs, the benefits of new varieties are immediate and directly applied (Saari et al. 1996).

The histopathology of the plant-pathogen interaction is key to understanding common bunt's underlying mechanisms of infection. Studies focused on the microscopic analysis of that interaction informs the basis for additional investigation of the physiological, molecular, genetic and biochemical aspects of pathogen development and host responses (Zhang et al. 2012a).

Resistance was the most powerful tool humanity had against common and dwarf bunt prior to the 1950s, and it must continue to play a part in controlling both. We know a great deal about the movement of the fungi in susceptible varieties, but our knowledge of resistance beyond theoretical genetics is limited. Although mapping studies have developed QTL and KASP markers for breeding purposes (Muellner et al. 2020b), further studies are required to elucidate the action of resistance genes and determine whether qualitative resistance alone provides durable host resistance. Such knowledge may allow for more effective pyramiding of genes in breeding, develop a deeper understanding of the utilization of marker-assisted selection (MAS) in these pathosystems, provide early diagnostic tools for breeding programs, and contribute to the body of knowledge on host resistance in cereals in general.

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Chapter 2:

A quantitative PCR assay method to assess relative resistance of winter wheat (*Triticum aestivum*) to common bunt (*Tilletia caries* and *T. laevis*)

Abstract

Common bunt, caused by two closely related species of *Tilletia*, is a reemerging pathogen of wheat that spreads asymptomatically within the plant until heading with the potential to devastate crops globally. Resistant cultivars continue to be one of the best management options, yet rating for resistance still requires growing winter wheat for months until it can be rated for disease at heading. Rating requires time, skill, and is somewhat subjective. A better method for early detection is needed. Previous research has resulted in several PCR-based assays for detecting common bunt, but they have largely focused on seed washes or destructive sampling and have not always used quantitative measures. This study aimed to use sequence-specific TaqMan quantitative PCR (qPCR) to quantify the relative amount of pathogen DNA within host tissue, thereby determining the extent of early infection in both destructively and non-destructively sampled tissues that might prove useful as an early diagnostic tool for predicting resistance in new cultivars. This study, additionally, aimed to corroborate molecular diagnostics of early qPCR infection with microscopic observation of infection within the same plant. Plants were inoculated with one of two races of common bunt, and subsets of plants were sampled and divided in two at different growth stages for microscopy observation, and DNA extraction and qPCR. Existing primers were used and a TaqMan probe developed, to assemble a multiplex qPCR assay capable of detecting 0.1 pg pathogen DNA. The polychromatic dye Toluidine blue O (TBO) in phosphate buffered saline (PBS) at a pH of 7.4 was used to dye coleoptile halves that had not yet developed chlorophyll, proving to dye fungal tissue magenta and plant tissue blue. Ultimately, while the qPCR assay is highly sensitive, there seem to be no significant differences in the course of infection prior to 46 dpi and highly race-specific reactions thereafter. At this time no correlation between early samples and disease outcomes can be established. This assay will be useful in future study of this pathosystem, however, to

routinely deploy this qPCR assay in resistance breeding further validation work is needed. The dyeing protocol, additionally, requires improvement before routine use is recommended.

Introduction

Common bunt (caused by two fungi; (*Tilletia caries* (A.P. de Candolle) L.-R. & C. Tulasne (syn. *T. tritici* (Bjerk.) G. Winter), and *T. laevis* J.G. Kühn (syn. *T. foetida* (Wallr.) Liro)) is a reemerging and destructive disease of wheat. Without resistance or seed protection, the fungi can infect the host plant early in its life cycle and grow asymptomatically until the heads are developed, at which point the fungi replace kernel tissue with balls of fetid spores (sori, bunt balls). As a lookalike of the quarantine pathogen *T. controversa* and as a detriment to grain marketability, it is important to protect plants through resistance breeding and to accurately diagnose infection. Diagnostics for common bunt infection rely on classic methods. In the field, this entails visually assessing stands for percent of bunted heads. This requires a fair amount of skill and experience to accurately accomplish, may vary from observer to observer, and when relied on in breeding programs may extend the generation time by several months. The industry standard for diagnosing infections in grain generally relies on the microscopic identification of spores by highly trained scientists, as well as weeks-long spore culturing in different environmental conditions to distinguish common and dwarf bunt fungi from each other.

Although not currently widely adopted for common bunt detection, polymerase chain reaction (PCR)-based methods have several advantages over classic disease rating. These assays use pathogen-specific DNA targets to detect the fungi in a sensitive, reliable, speedy, and potentially pre-symptomatic manner that requires little to no specialized training in common bunt morphology or symptomology. Real-time PCR (qPCR) advances PCR-based methods, allowing the development of an assay for the amplification and quantification of specifically bunt DNA. TaqMan qPCR uses species-specific primers to cleave DNA fragments from the genomic DNA and uses fluorescent tagging of these fragments to quantify the original amount of DNA. This assay has been well and thoroughly adopted in plant pathology for a number of applications

(Mirmajlessi et al. 2015; Schena et al. 2004; Schaad and Frederick 2002). It has been used in other pathosystems to correlate the amount of fungal pathogen DNA in host samples to the severity of infection or extent of resistance as classically rated. For example, Fusarium head blight DNA quantities have been correlated to resistance in wheat (Horevaj et al. 2011; Burlakoti et al. 2007), tan spot and glume blotch DNA in wheat (Abdullah et al. 2018b), Aphanomyces root rot and root rot in alfalfa (Vandemark and Barker 2003; Vandemark et al. 2002), eyespot in wheat (Meyer et al. 2011), loose smut in barley and wheat (Wunderle et al. 2012), and many other pathosystems have been likewise studied. Several qPCR methods have also been published for detection of common bunt of wheat (Forster et al. 2021; Orgeur 2021; Zgraja et al. 2016; Zouhar et al. 2010; McNeil et al. 2004).

However, the qPCR methods published for common bunt currently use standard curves to assess the quantity of pathogen DNA, often either in seed lots or destructively sampled host tissue rather than non-destructively sampled host tissue. When diagnosing a large volume of samples, it may be tedious and resource-heavy to include a dilution series for a standard curve in every plate. Including a standard curve may use 5-10% of available wells in a qPCR plate. Recently, qPCR assays involving an endogenous host control have been developed that reduce the resources, time, and mathematics required to quantify DNA while improving the reliability of the data by normalizing results against variation in initial DNA quantity (Admassu-Yimer et al. 2019; Acevedo et al. 2010). While other assays involving sequence characterized amplified region (SCAR) markers, and marker-assisted selection (MAS) have been developed to assess the resistance of host material, they have different advantages and disadvantages. SCAR markers can be very useful for pathogen detection, but so far their use in PCR-based assays has been limited to single-plex SYBR Green assays (Xu et al. 2020; Yao et al. 2019; Zhang et al. 2012b). MAS can be used to identify known resistance markers or genes but cannot provide a determination of fungal spread within plant tissue.

Although qPCR is highly efficient, high throughput, specific, and requires minimal training, it cannot be used for visual analysis. A number of microscopy studies have been published on the progression of common bunt in susceptible reactions using

a number of microscopy techniques (Ren et al. 2021; Gaudet et al. 2007; Swinburne 1963; Churchward 1940; Woolman 1930). None of these paired molecular techniques with microscopy techniques. In the absence of molecular techniques, microscopy studies using non-species-specific techniques may observe non-target fungi such as endophytes. Microscopy also requires the production of many sections, only some of which will be fit for observation, allowing for the potential to miss infection events. In pairing microscopy with molecular techniques, a study could be produced that could cross-validate descriptions of the location and amount of common bunt colonization as evidenced by DNA, and microscopic observations on the histopathology of the disease.

To reduce the specialized-skill requirement and standardize ratings of infection, and to improve breeding timelines, a qPCR-based approach to common bunt diagnostics provides an alternative to the current approach, and this may be more accurate and reproducible. This study aimed to (i) modify existing molecular approaches to develop an assay capable of quantifying the amount of common bunt DNA relative to the amount of host wheat DNA in a sample to (ii) determine the extent of earlier infection in two different host genotypes challenged with two different common bunt races and (iii) compare results to microscopic observations to cross-validate the description of early infection. The final objective was to (iii) investigate whether non-destructive samples taken earlier in the plant life cycle could be used as a tool for resistance breeding, to shorten the breeding cycle in the absence of QTL.

Materials and Methods

Experimental Design

Three replications of this experiment were conducted in controlled environmental conditions over the period from December 2019-July 2021. To study the difference between compatible and incompatible reactions, in each replication two different cultivars with different susceptibility patterns were challenged with each of two races of common bunt. Specifically, *Triticum aestivum* subsp. *aestivum* cv. 'Heines VII' (carrying *Bt0,* the absence of resistance) and *T. aestivum* subsp. *aestivum* cv. 'Yayla 305' (carrying *Bt8,* a source of broad-spectrum and durable resistance gene) were both challenged with both *Tilletia laevis* race L-18 and *T. caries* race T-34. Of these, only the

combination of 'Yayla 305' and *T. caries* race T-34 has been reported to be incompatible, showing less than 7% disease at heading (Matanguihan and Jones 2011). Heines VII has been used previously as a susceptible check, showing greater than 70% bunted spikes across years (Goates 2012).

Given the goal of the study to describe the movement or halting of the common bunt fungus throughout the wheat life cycle, five growth stages (GS) were selected for study;

1. Pre-emergence (Zadoks 5, Feekes 0)
2. Emergence (Zadoks 7-9, Feekes 1)
3. 2nd leaf emerged (Zadoks 12, Feekes 1)
4. 2nd node detectable (Zadoks 32-39, Feekes 7)
5. Maturity (Zadoks 85-92, Feekes 11.2-11.3)

The first four growth stages were sampled for DNA extraction and further analysis with qPCR. The fifth growth stage was classically rated for disease phenotype (as percent of bunted kernels out of all kernels on a plant) at maturity.

Each of the three replications of the experimental trial consisted of at least 54 plants per combination of cultivar and race, of which 4-9 plants of each were sampled at each growth stage along with 2-3 plants of each control group at each growth stage. The number of plants sampled was dependent on germination of seed.

Seed and Inocula Source

Wheat (*Triticum aestivum*) seed of two varieties was obtained from the National Small Grains Collection (NSGC) in Aberdeen, ID. PI 178210 (cv. 'Yayla 305'), a pureline winter wheat bred from a Turkish landrace was used as the *Bt8* differential. The *Bt0* differential cv. 'Heines VII', a pureline hard red winter wheat was also used. NSGC seed was increased through 2019 and 2020 by growing out plants in Conviron growth chambers (Conviron, Pembina, ND, USA) and greenhouse facilities at the University of Idaho Research and Extension Center in Aberdeen, ID.

Previously identified and collected isolates of *T. laevis* race L-18 and *T. caries* race T-34 were used for inoculations. L-18 sori were sourced from the collections of Dr. Jianli Chen (University of Idaho wheat breeding program), which had been grown on

susceptible wheat cultivar 'Rio Blanco' in Aberdeen, Idaho. T-34 sori were sourced from the original collection of Dr. Janet Matanguihan in Pullman, Washington.

Culture Preparation

T. laevis and *T. caries* isolates were surface sterilized and incubated on 2% water agar (Difco® Bacto® Agar, Becton, Dickinson and Company, Sparks, MD) in full dark at 15°C for 3-5 days. Germinated hyphae were transferred to 3.9% potato dextrose agar (Becton, Dickinson and Company, Sparks, MD) and cultured in the same conditions for at least 2 weeks.

Inocula Preparation

Sori of isolates *T. caries* race T-34 and *T. laevis* race L-18 were stored dry in a sealed sterile tube at room temperature until use. Sori were sterilized in 5% household bleach for 20 seconds, rinsed 3 times in sterile water for 20 seconds each rinse, and dried gently with a sterilized Kimwipe. 4 sori were crushed and suspended in 750 µl of 0.5% methyl cellulose solution (Carolina Biological Supply Company, Burlington, North Carolina) for each lot of 100 seeds, amounting to a spore suspension of concentration 25,000 spores/µl.

Inoculation

Inoculation followed a modified protocol described by Goates (2012). The modified protocol follows the preparation of an aqueous solution of 0.5% methyl cellulose (Carolina Biological Supply Company, Burlington, North Carolina), which may be stored at room temperature in clear glass. Preparation of the methyl cellulose solution involved heating the water and slowly adding the powdered methyl cellulose while stirring.

Seeds were immediately inoculated with the inocula prepared as previously described without prior surface sterilization. Seeds were allowed to soak in the inoculum solution for 1.5-2 hours at room temperature in light conditions. After the soaking period, seeds were placed 5 cm. deep in either a square 4 inch pot (100% of plants for Trial 1 and 50% of plants for Trials 2 and 3) or white Ray Leach Low Density

Cone-tainer™ (Steuwe & Sons, Inc., Tangent, OR) (50% of plants in Trials 2 and 3) filled with a moistened blend of 40 parts peat moss, 40 parts sand, 40 parts vermiculite and 1 part Osmocote™ Outdoor and Indoor Plant Food fertilizer. Seeds were germinated in a Conviron growth chamber set to 12 hour 10 °C full light periods with 5 °C full dark periods.

For each trial, a set of mock inoculated seeds of each variety was also included as a negative control. Mock inoculation consisted of vortexing and soaking the seed in the methyl cellulose solution for the same duration as the seeds inoculated with the teliospores. They were then grown in the same conditions and sampled in the same way at the same times as the teliospore-inoculated plants.

Growth Conditions

After growing past Feekes 1 (Zadoks 12) in germination conditions, the plants were vernalized for 8 weeks with 8 hours of full light at 8 °C and 16 hours of full dark at 5 °C. At the end of 8 weeks, the temperatures were gradually raised to greenhouse conditions. For Trial 1, this was done by increasing the temperature by 3°C weekly up to a final temperature of 27°C. For Trials 2 and 3, after observing heat stress in Trial 1, conditions were gradually warmed to a 16-hour full light period at 21°C, with an 8-hour dark period at 10°C. Plants were watered with tap water (hardness >180 mg/liter) on a regular schedule. No additional fertilizer was applied.

Sampling

Sampling was done using sterilized equipment and surfaces at the University of Idaho Aberdeen Research & Extension Center. Each growth stage was sampled using techniques best fitting for the plant tissues at that stage. After sampling, samples were preserved at -20 °C for up to 2 weeks and then -80 °C until DNA extraction.

For pre-emergent seedlings (Zadoks 5, Feekes 0, Figure 1), plants were gently removed from the Cone-tainer soil and if necessary, briefly and carefully washed in still Millipore water to preserve, as much as possible, the fungal mass on the coleoptile. Seedlings were then removed from the seed and divided into a root sample and a coleoptile sample. The root sample was preserved in a clean microcentrifuge tube. The

coleoptile was bisected longitudinally, with one half preserved in a clean microcentrifuge tube for freezing and eventual DNA extraction and the other reserved for microscopic observation. Seedlings at emergence (Zadoks 11, Feekes 1, *Figure 2*) were sampled much the same as pre-emergent seedlings.

Figure 1: A pre-emergent seedling, representing the first sampling growth stage.

Figure 2: An emerging seedling, representing the second sampling growth stage.

Seedlings at the 2-leaf stage (*Figure 3*) were gently removed from the Cone-tainer soil, and the roots cut with clean scissors 3 cm. below the seed. The remaining soil was carefully scraped away, and the roots were then swirled in still Millipore water until clean. Seed coat was then discarded, and roots cut away from the plant and frozen.

After this, the crown along with the 1 cm. of developing tissue surrounding the crown was cut from the stem. The crown was bisected longitudinally, and one half frozen. Left with the stem, consisting of the first leaf and desiccating coleoptilar sheath surrounding the second leaf, the leaves were cut away 3 cm. above the point at which they diverged from each other (where the second leaf fully emerges). The first leaf was then gently pulled away from the second, bisected longitudinally, and one half frozen. The second leaf was then bisected longitudinally, and one half frozen.

Figure 3: A plant at the two-leaf stage, representing the third growth stage.

Plants at the older growth stage, Zadoks 32-39 (Feekes 7) were removed from the soil and their roots cut away with clean scissors (*Figure 5*). The crown, often tough and woody at this stage, was cut from the tiller bases and bisected. If the crown was larger than 6 mm^3, it was cut into smaller portions representatively and one half frozen. The three most developed tillers were then selected from the remaining plant. For each tiller, the first viable leaf was pulled away from the tiller, cut to roughly 7 cm. from the base, bisected longitudinally, and one half frozen. The remaining stem was then dissected. The first node was removed, bisected longitudinally, and one half frozen. The second node was then dissected the same. At this point, tillers were developmentally different from each other and thus the entire stem was bisected longitudinally to better visualize the developing tissues inside the stem. Third nodes, and if present fourth nodes and developing heads were cut away from the stem and one half frozen.

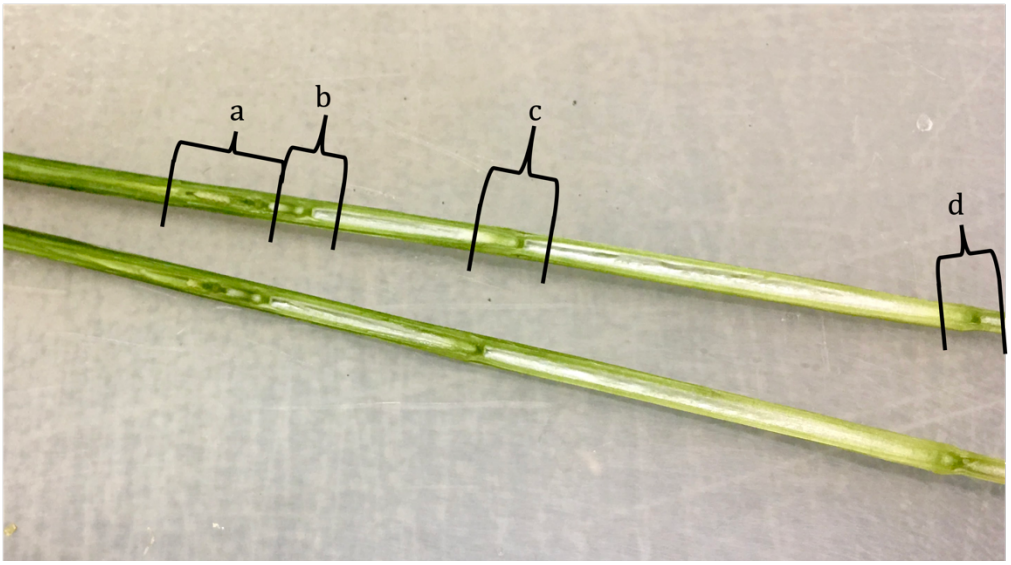


Figure 4: Stem dissection, areas in brackets were excised for DNA extraction. a; developing head. b; third node. c; second node. d; first node.

Figure 5: A plant at the second node stage, representing the fourth growth stage.

Rating Percent Disease Incidence

Classical disease rating has involved visually assessing the proportion of bunted heads in a stand and relying on necessarily arbitrary cutoffs to determine resistance or

susceptibility. As discussed in the literature review chapter of this thesis, this is an unsatisfactory approximation of the range of resistance responses in wheat. To add precision to this measurement, percent disease incidence (% DI) was recorded as both per treatment % DI (infected plants out of the total stand) and per plant % DI (sori out of all developed ovaries).

Percent disease incidence of a plant was used to refer to the proportion of sori (infected kernels) out of all developed "kernels" present on a plant. Plants were scored for number of infected heads out of the total number of heads. After this initial rating, individual spikelets were opened to tally the number of seeds and number of sori. Disease incidence (% DI) for each plant was then calculated as:

$$\%DI = \frac{s}{\sum s + k}$$

Equation 1

where *s* demarcates the number of sori total across spikes and *k* the number of healthy kernels across spikes.

Percent disease incidence per treatment was calculated as the ratio of diseased plants (plants with at least 1 sorus, p_d) out of all plants in the treatment (p_t):

$$\%DI = \frac{p_d}{p_t}$$

Equation 2

DNA Extractions

DNA was extracted from both frozen mycelia and healthy wheat tissue for fungal and host DNA standards, respectively. Total genomic DNA was also extracted from frozen halved inoculated host tissues using the Norgen Biotek Plant/Fungi DNA Isolation Kit (Cat. 26250, Norgen Biotek Corp., Thorold, ON, Canada). Sample cells were disrupted using a Spex Sample Prep Geno/Grinder (Spex SamplePrep, Metuchen, NJ) and two 2 mm. steel beads. The samples were ground to a paste in their tubes, using the GenoGrinder set to 1500 rpm for 1-5 minutes as necessary, with sets of 5 minutes repeated as necessary for woodier tissue. Ground samples were then frozen again for at least one day. After this, the Norgen kit was used as directed.

Pure cultured mycelium DNA was extracted after freezing at -80°C for at least 1 week. Mycelium cells were disrupted by bashing thawed tissue in 600 µl Lysis Buffer (Norgen Biotek Plant/Fungi DNA Isolation Kit, Cat. 26250, Norgen Biotek Corp., Thorold, ON, Canada) with 2 mm steel beads on the Spex Sample Prep Geno/Grinder. The suspended tissue was shaken at 1500 rpm for 5 minutes, until the cells were evenly distributed in the Lysis Buffer. After cell disruption, the Norgen kit was used as directed.

DNA Dilutions

DNA concentration and quality were read on a Biotek Synergy H1 Microplate Reader (BioTek Instruments, Winooski, VT). Samples were then diluted with 1X Tris to either 10 ng/µl or 5 ng/µl depending on the concentration of the extract and stored at -20°C.

Preliminary Tests

Primers developed by Zgraja et al. (2016) (see Table 1) to amplify fungal pathogen DNA of the common bunt pathogens were tested with SYBR Green and BioRad Precision Melt Supermix with a final melt curve to assess PCR products for dimerization. Each well contained 10 µl Precision Melt Supermix, 1 µl of each 2 µM primer, 6 µl molecular biology grade water, and 2 µl DNA template totaling 20 ng genomic DNA of pure cultured L-18 mycelium. Cycling conditions were a 2 minute hold at 95 °C, then 40 repeats of 95 °C for 10 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds followed by a plate read. After 40 repeats, the melt curve analysis was developed by increasing the temperature from 70 °C to 90 °C in 2 °C increments, with a plate read every 5 seconds. This produced both a series of fluorescence reads (curves) and a melt curve. Fluorescence curves were visually assessed for Ct value stability, curvature, and strength of amplification. The melt curve was visually assessed for the solitude of the peak. These being satisfactory, the Zgraja et al. (2016) primer-probe combination was tested in a multiplex qPCR using fungal DNA of the common bunt pathogen and uninoculated wheat DNA and amplified as expected.

Primers designed to amplify common reference genes with stable expression in wheat were selected from the work of Wei et al. (2015) (see Table 1) and were tested

by a SYBR Green BioRad Precision Melt Supermix in both a non-template molecular biology grade water as a control and a series of samples representing different tissues and growth stages from this project. Each 20 µl reaction volume consisted of 10 µl Precision Melt Supermix, 1 µl of each 2 µM primer being tested in that well, 6 µl molecular biology grade water, and 2 µl DNA template totaling 20 ng genomic DNA. Cycling conditions consisted of a 2 minute hold at 95 °C, then 40 repeats of 95 °C for 10 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. After the 40th repeat, the plate was held at 4 °C. This produced a series of fluorescence curve reads, which were visually assessed for variation in Ct values across sample types and strength of amplification. The PCR products were then run in duplicate on a 1.5% agarose gel at 90 volts for 1.5 hours to verify the size of the amplicon and the number of PCR products. After selecting the AB181991 primers published by Wei et al. (2015), it became necessary to design a probe. The probe for this primer pair was designed using Primer3Plus (Untergasser et al. 2012) with the source sequence from the National Center for Biotechnology Information (NCBI), and flanking primer sequences from Wei et al. (2015). Default settings were used, other than an adjustment to the GC% to a minimum of 30, optimum of 40, and maximum of 50. The resulting sequence (Table 1) was used to develop the probe AB181991-Pr tagged with [HEX] and [BHQ1] (Eurofins Genomics). This probe was validated against DNA from multiple wheat tissue types and DNA from pure fungal cultures, and the concentration of the probe in the reaction was optimized for use in the qPCR conditions specific to the Universal Express qPCR SuperMix (Invitrogen, Waltham, MA).

Primers were consistently used in multiplex as 1 µl at a 10 µM concentration. This was selected given the standard practices of qPCR in plant pathology and confirmed through repeated use in multiplex conditions. Each 25 µl reaction well consisted of 12 µl Universal Express SuperMix (Invitrogen, Waltham, MA), 5 µl molecular biology grade water, 1 µl of each wheat as well as fungal primer (10 µM), 1 µl of wheat probe (variable concentrations), 1 µl of fungal probe (variable concentrations), and 2 µl template DNA totaling 20 ng genomic DNA. Real-time PCR thermal cycling was conducted using Bio-Rad CFX96 (Bio-Rad Laboratories, Hercules, CA). Cycling conditions were described by the manufacturer for Universal Express

SuperMix: 50 °C for 2:00 mins, 95 °C for 2:00 minutes, 60 °C for 1:00 minute followed by a plate read and 40 cycles of amplification then a hold at 4 °C.

Probes were tested at three concentrations; 5 µM, 2 µM, and 1 µM. Multiplex conditions were continued as previously described, and the results were visually assessed for consistency of Ct values in different reactions of the same sample and amplification strength. The strongest and most consistent concentration was selected. In the case that two or more concentrations were comparable, the middling concentration was selected to balance material use and efficiency.

After confirming the performance of both the selected fungal primers and probes, and the wheat primers and probes in multiplex with each other, the primers and probe selected from Zgraja et al. (2016) and Wei et al. (2015)'s primers and the herein developed probe based on the AB181991 sequence were used for all qPCR reactions.

Limit of Detection

After optimizing concentrations of primers and probes, the limit of detectable DNA amounts was tested for each oligo set. A 10-fold dilution series was produced consisting of 10 ng/µl, 5 ng/µl, 0.5 ng/µl, 0.005 ng/µl, 0.0005 ng/µl, and 0.00005 ng/µl. Each respective dilution solution was used at 2 µl to create a series of 20, 10, 1, 0.1, 0.01, and 0.001 ng genomic DNA in each respective well. Each 23 µl well contained 12 µl Universal Express SuperMix (Invitrogen, Waltham, MA), 8 µl molecular biology grade water, 1 µl of both 10 µM primers, 1 µl 2 µM probe, and 2 µl of the respective genomic DNA dilution. Cycling conditions were: 50°C for 2:00 mins, 95°C for 2:00 minutes, 60°C for 1:00 minute followed by a plate read and 50 cycles of amplification then a hold at 4°C. The default software settings were used to calculate the threshold and Ct values.

qPCR Conditions

Each sample was run through a multiplex PCR assay intended to amplify the host DNA and any present pathogen DNA. To achieve this, 2 sets of oligos were used – one specific to wheat with a HEX-labeled probe and the other specific to *T. caries*, *T. laevis*, and *T. controversa* with a FAM-labeled probe (Table 1). All samples were run in duplicate, with

both positive (pure wheat DNA and, separately, pure *T. laevis* DNA) and negative (molecular biology-grade water) controls performed in duplicate with each plate. Primers (Table 1) were ordered from Eurofins Genomics (Louisville, KY, USA). Each 25 µl reaction well consisted of 12 µl Universal Express SuperMix (Invitrogen, Waltham, MA), 5 µl molecular biology grade water, 1 µl of each wheat as well as fungal primer (10 µM), 1 µl of wheat probe (2 µM), 1 µl of fungal probe (2 µM), and 2 µl template DNA totaling 20 ng genomic DNA. Real-time PCR thermal cycling was conducted using Bio-Rad CFX96 (Bio-Rad Laboratories, Hercules, CA). Cycling conditions were: 50°C for 2:00 mins, 95°C for 2:00 minutes, 60°C for 1:00 minute followed by a plate read and 40 cycles of amplification then a hold at 4°C. The default software settings were used to calculate the threshold and Ct values. Samples were considered positive for common bunt if the Ct values were lower than a cycle below the negative control for the plate, or the 0.1 pg limit of detection if no controls provided any amplification. This provided stability between plates.

Table 1. Sequences of primer-probe sets utilized to amplify fungal pathogen DNA from common bunt -infected wheat tissue

Name	Gene Accession #	Sequence	Amplicon length (bp)	Target	Source
AB181991	AB181991	**F:** AGCGGTCGAACAACTGGTA	101	wheat ACT	Wei et al. (2015)
		R: AAACGAAGGATAGCATGAGGAAGC		wheat ACT	Wei et al. (2015)
		P: [HEX]-TGAGCCACACTGTTCCAATC-[BHQ1]		wheat ACT	this work
Til122	HQ317580	**F**: ACCCATTGTCTTCGGACTTG	140	*Tilletia* spp. ITS region	Zgraja et al. (2016)
Till262		**R**: GGTGCGTTCAAAGATTCGAT		*Tilletia* spp. ITS region	Zgraja et al. (2016)
Til175		**P**: [FAM]-CTTGGTTCTCCCATCGATGAAGA-[BHQ1]		*Tilletia* spp. ITS region	Zgraja et al. (2016)

Note: F – forward primer, R – reverse primer, P – probe

Primer Efficiency Calculations

Efficiencies for each of the two primer sets used were calculated based on an average of the runs of the 10-fold dilution series performed. For each run of the dilution, the average Ct of each diluted sample was calculated and plotted against the log of the sample quantity. The slope of the regression line was calculated using Excel Version 16.59's "SLOPE" function, and the R^2 value using the program's "RSQ" function. The efficiency was then calculated using *Equation 3*;

$$E_{primer} = (10^{\frac{-1}{slope}} - 1) \times 100$$

Equation 3

The efficiencies were separately calculated for at least two runs of the standard curve, and then averaged for use in relative DNA quantification.

Quantification

Ct values were recorded for each well after each run and were averaged between the two duplicates to determine an average host Ct (hCt) and an average pathogen Ct (pCt). Controls of a known 0.001:1 mix of pure fungal pathogen DNA and pure wheat DNA were run multiple times (seventeen times) to determine an average control value for both hCt and pCt.

The Pfaffl equation (*Equation 4*) was then used to calculate the relative amount of bunt DNA in a sample (Pfaffl 2001).

$$ratio = \frac{(1 + E_{bunt})^{\overline{(pCt_{control} - pCt_{sample})}}}{(1 + E_{wheat})^{\overline{(hCt_{control} - hCt_{sample})}}}$$

Equation 4

Data Analysis

After calculating the relative amount of bunt DNA within host samples, analysis was done using SAS v. 9.4. Only data for inoculated groups was considered, as the controls demonstrated a lack of contamination. Outliers calculated to have above 60% relative pathogen DNA were excluded, due to the biological improbability based on observation

of the tissue and previous work demonstrating the lower density of hyphae in tissue (Hansen 1958; Woolman 1930). To analyze data that contained multiple 0 values due to unsuccessfully inoculations or incompatible reactions, a value of 0.0000001 was added to all relative pathogen DNA values. Data was sorted according to trial replication, race of fungal inoculum, cultivar, and growth stages 1-4. PROC MEANS was then employed to create a table of mean relative pathogen DNA values for each replication/race/cultivar/growth stage group. This table of means was then modeled using PROC GLIMMIX to log transform the data and develop a generalized linear mixed model with a random complete block design. The random effect was set as the interaction between trial replication, bunt race, wheat cultivar, and growth stage. The fixed effects were cultivar, bunt race, growth stage, and all combinations of the three factors. The outputs of this model were assessed for significance by studying the p-values and effect sizes of the overall model and parameters.

Spearman correlations between relative pathogen DNA and disease incidence at maturity were done using the PROC CORR function in SAS v. 9.4 with the "Spearman" option.

Microscopy

A 0.01% weight/volume Toluidine blue O (TBO) dye was prepared by dissolving 0.01 g TBO (CAS 92-31-9, Thermo Scientific™, Waltham, MA, USA) in 0.1 M phosphate-buffered saline (PBS) (CAS 7647-14-5, 7447-40-7, Fisher BioReagents™, Pittsburgh, PA, USA) prepared with deionized water.

The pre-emergent coleoptiles in the first replicated trial were cleaned and separated into the basal portion with meristematic tissue and the shoot portion. Given that the plants had not yet developed chlorophyll, no clearing was necessary.

Coleoptiles were allowed to float in a petri dish of dye for 4 minutes to aide in further sectioning, after which they were rinsed in Millipore water. Tissue was placed in a shallow petri dish of Millipore water until sectioned. The shoot portion was then cut from the meristematic portion consisting of the lower ~50 mm of the coleoptile.

The meristematic portion was then hand-sliced longitudinally with a double-edged razor blade and sections were carefully placed on a clean slide with the tip of a

dissecting needle. Sections on the slide were covered in a drop of dye and allowed to dye for 2-3 minutes depending on the thickness of the section.

The shoot portion was cut into cross sections with a double-edged razor blade, and cross sections were likewise dyed on the slide for 2.5-3 minutes depending on section thickness.

Once dyed 40-90 µl of Millipore water, depending on the number of sections on the slide, was used to flood the sections and dilute the dye. Sections were rearranged with the tip of a dissecting needle, if necessary. A clean cover slip was carefully placed on the sections.

Slides were viewed with an Olympus CX41 at 40X, 100X, and 400X magnifications. The sections were observed for the presence or absence of dyed mycelia, and for description of the intra- or intercellular progression through observed tissues. Photos of were taken with an iPhone 6S through the ocular and cropped to frame the tissue. Photos were edited in iPhoto for contrast, white balance, and exposure when necessary. Photographs were not taken with scale bars.

Results

Assay Development

A TaqMan probe was designed in association with the primers and probes published by Zgraja et al. (2016) for the *Tilletia* common and dwarf bunt ITS region and primers published by Wei et al. (2015) for the wheat *β-actin* gene. Both sets of primers and probes produced a single PCR product. The *β-actin* primers amplified a single ~100 bp product (*Figure 6*), and the bunt ITS primers produced a single melt peak (*Figure 7*). The set of *β-actin* primers and probe was tested at three concentrations (5, 2, and 1 µM) to determine the concentration for the multiplex assay. Among the three concentrations, 2 µM was determined to be the concentration most useful at the lowest amount (*Figure 8*). The AB181991 *β-actin* primers and probe were then used in multiplex with primers and probes developed by Zgraja et al. (2016) for common and dwarf bunt to detect trace amounts of common bunt DNA in infected wheat tissue. The common bunt ITS detection was found to be 96% efficient (*Figure 9*), and the wheat *β-actin* 81% (*Figure 10*) when cycled at the conditions specified for the Universal Express

SuperMix (Invitrogen, Waltham, MA). Using these conditions, the primer-probe sets were able to detect as little as 0.1 pg fungal pathogen DNA (*Figure 9*). Due to the incongruency between the two efficiencies, quantification of relative fungal pathogen DNA relied on Pfaffl's modification of the $2^{\Delta\Delta Ct}$ method.

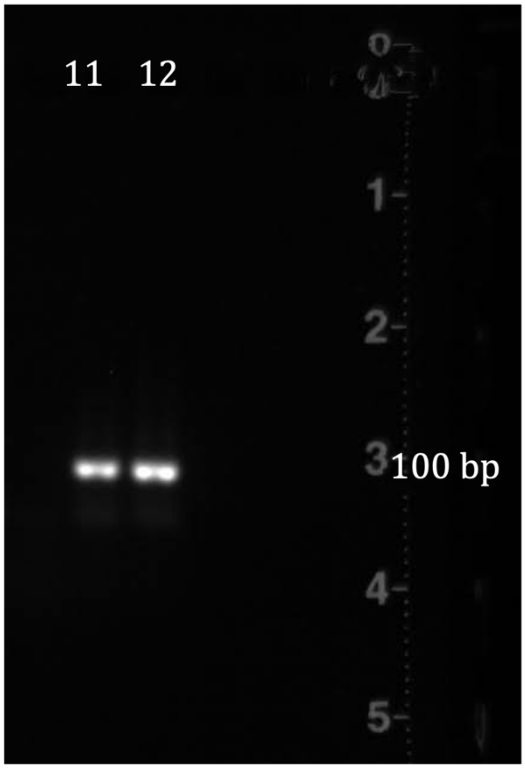


Figure 6: Visualization of the product of wheat β-actin gene primers AB181991-F/R on 1.5% agarose gel. 11 and 12 β-actin amplicon of uninoculated wheat. [Not pictured: 1, 1000 bp ladder. 2-10, miscellaneous primer sets]

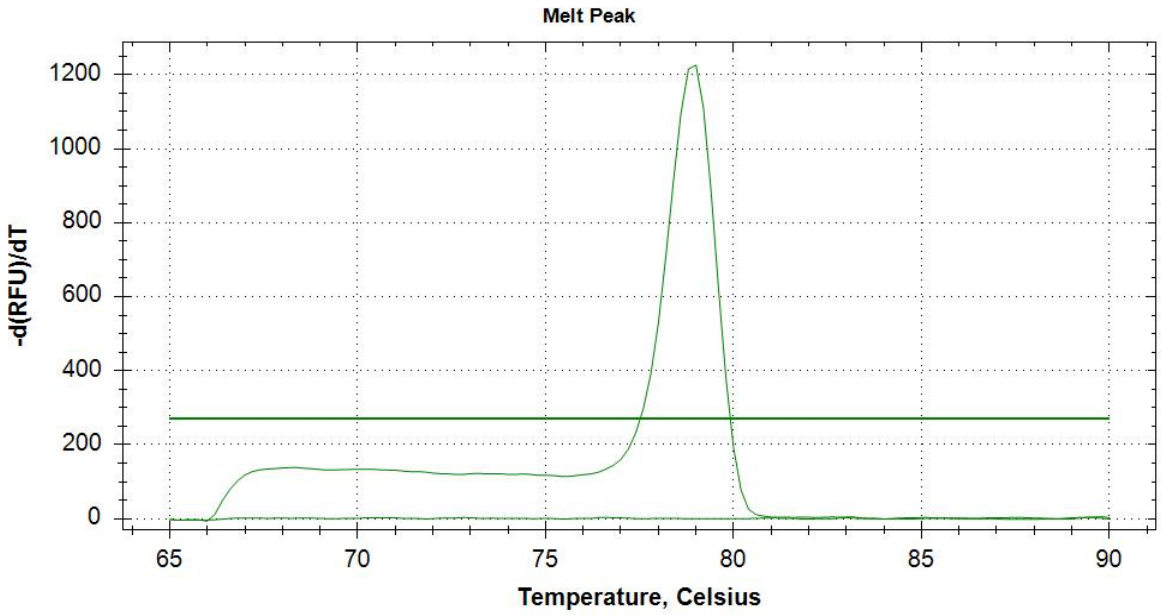


Figure 7: Melt peak for Til122-F, Till262-R, and Til175-P amplification of pure T. laevis DNA.

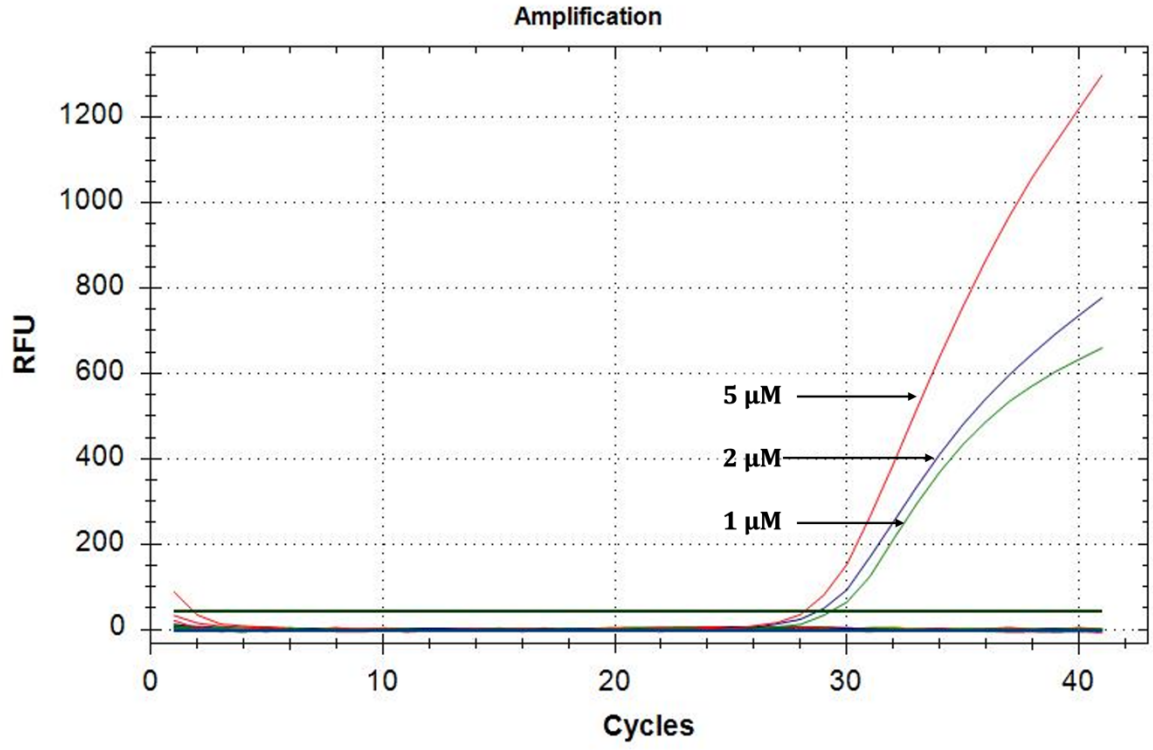


Figure 8: AB181991-Pr tested at three concentrations for optimization of use in the multiplex assay; red (5 µM), blue (2 µM), and green (1 µM).

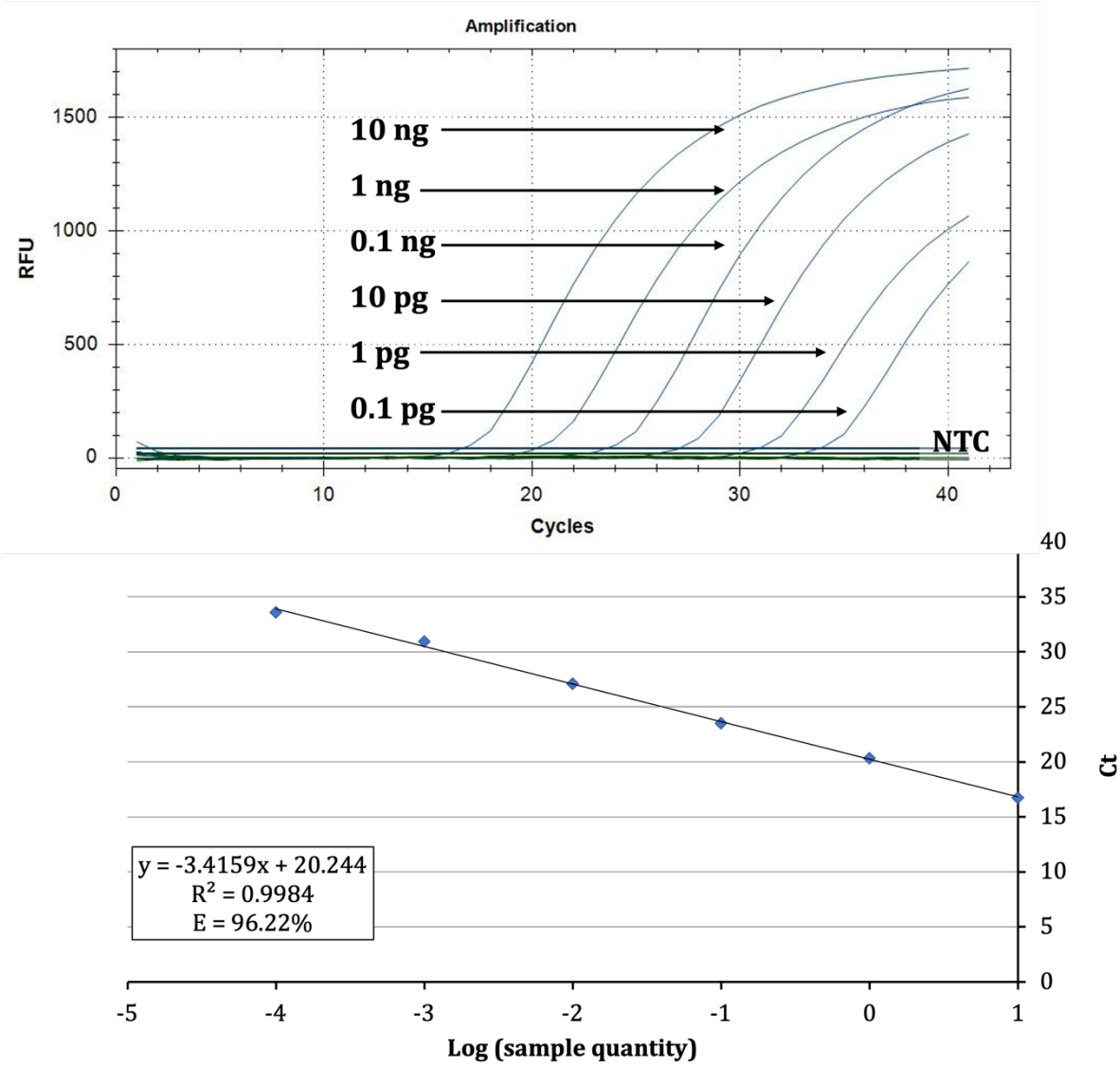


Figure 9: Amplification profiles (upper) and standard curve (lower) from the 10-fold dilution of T. laevis race L-18 DNA using the forward primer Til122-F, reverse primer Till262-R, and TaqMan probe Til75-P set. NTC = no template control. E = PCR efficiency

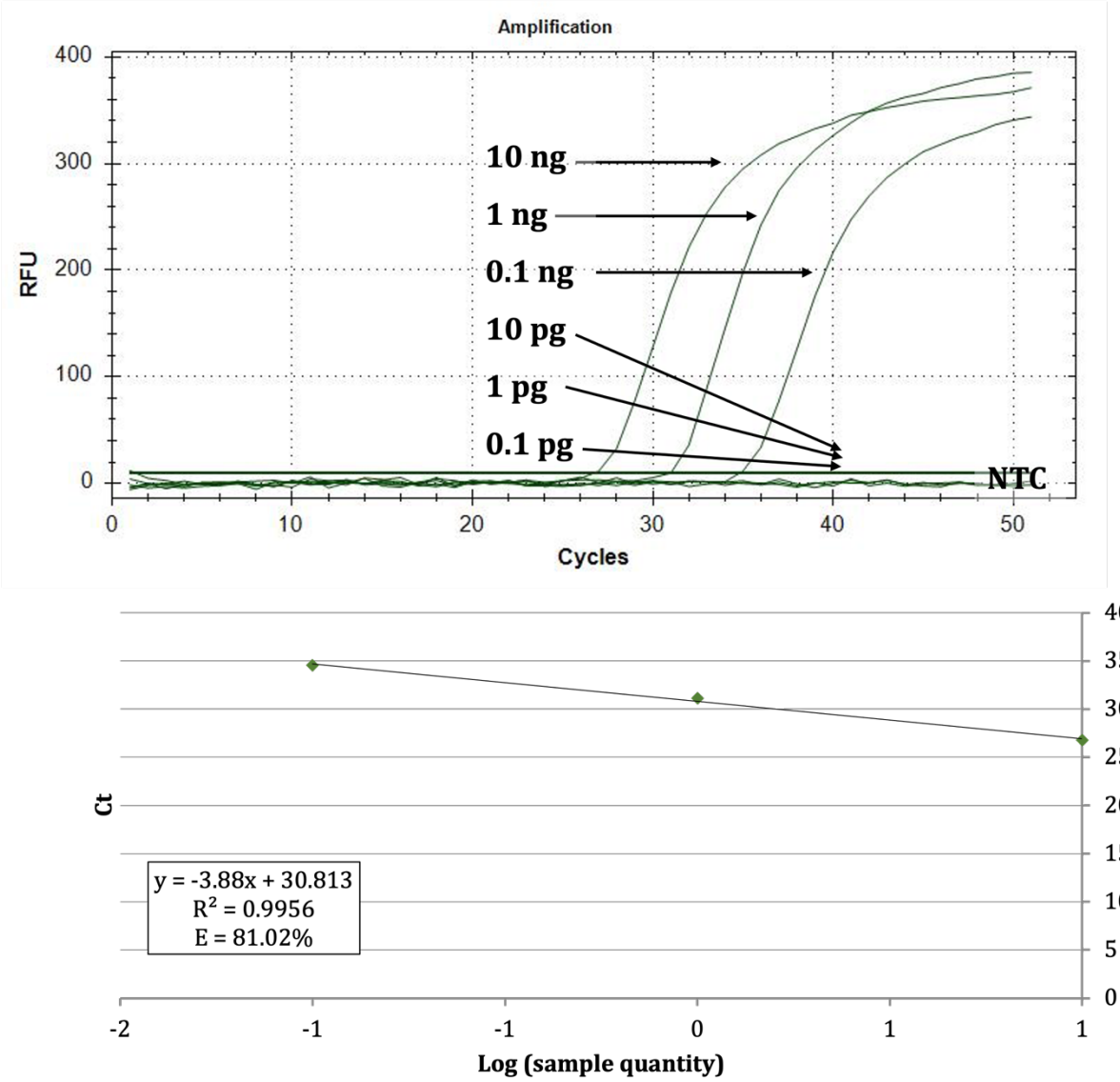


Figure 10: Amplification profiles (upper) and standard curve (lower) from the 10-fold dilution of uninoculated T. aestivum DNA using the TaqMan AB181991 primer and probe set. NTC = no template control. E = PCR efficiency

The ability of the assay to detect trace amounts of fungal pathogen DNA in wheat host DNA samples was tested by an emulation of an infected sample, containing a 0.001:1 ratio of pure cultured *T. laevis* race L-18 DNA in uninoculated wheat DNA, for a total of 0.02 ng pathogen DNA in 20 ng of DNA per well. In all cases, the 0.02 ng fungal pathogen DNA amplified at an average of 24 cycles which is consistent with the standard curves obtained for the pure fungal pathogen DNA alone. The 20 ng of wheat DNA amplified at an average of 26 cycles.

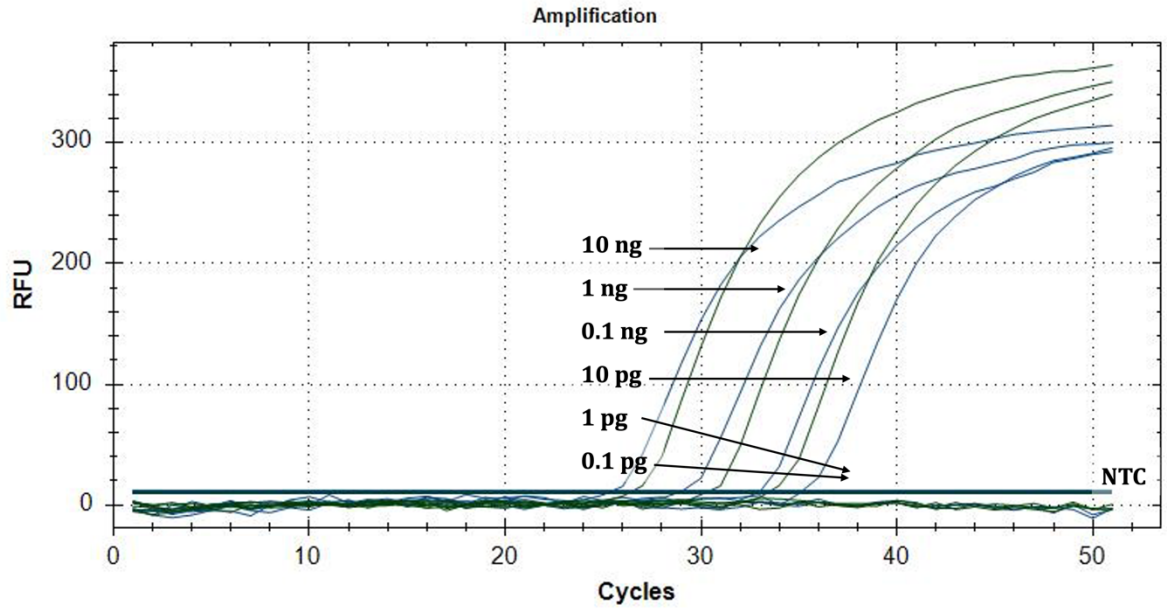


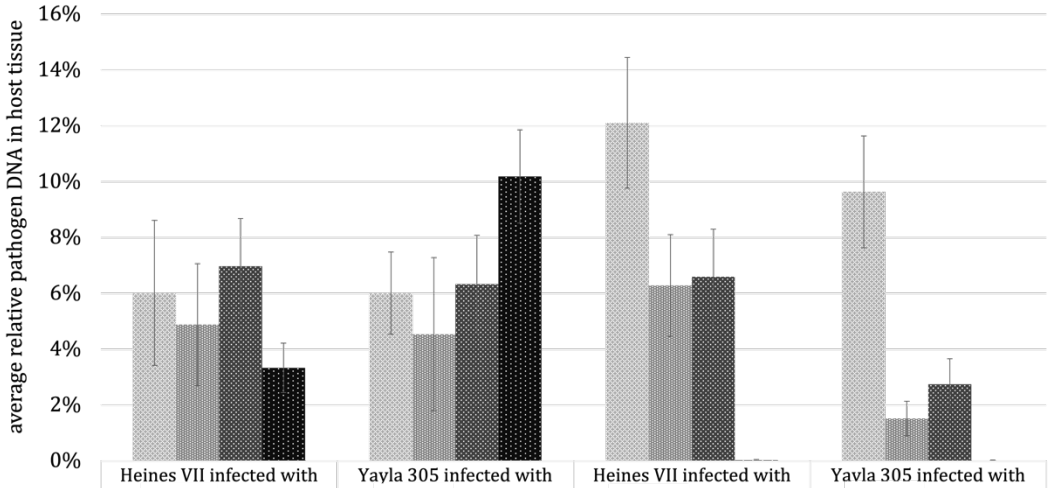
Figure 11: Amplification of a dilution series of the 0.001:1 pathogen: host DNA in which labeled quantities represent total combined DNA. Green: HEX-labeled AB181991 wheat response. Blue: FAM-labeled Tilletia common bunt response. NTC: No template control.

Determination of relative fungal pathogen DNA contents in early incompatible reactions vs. compatible reactions

In plants sampled before 46 days after inoculation, there were no statistically significant differences between the average relative fungal pathogen DNA contents (*Figure 12*). All treatment groups were positive for common bunt, and averages within the pre-emergence stage ranged from 6-12% relative fungal pathogen DNA within a sample with a p-value of 0.7958. At the emergence stage, averages ranged from 2-6% relative fungal pathogen DNA within a sample with a p-value of 0.4340. Between 21-46 days post-inoculation, at the two-leaf stage, differences in infection were likewise insignificant statistically, ranging from 3-7% relative fungal pathogen DNA within a sample with a p-value of 0.8276.

At the 4th growth stage, the stage at which the second node becomes visible between 119-151 days post-infection, the differences between treatment groups were statistically significant with a p-value of 0.0042 and a range of 0-10% relative pathogen

DNA content. The incompatible interaction had no detectable DNA, while two of the three compatible interactions had detectable DNA (*Figure 12*).



	Heines VII infected with *T. laevis* race L-18	Yayla 305 infected with *T. laevis* race L-18	Heines VII infected with *T. caries* race T-34	Yavla 305 infected with *T. caries* race T-34
Stage 1: Pre-emergence	6%	6%	12%	10%
Stage 2: Emergence	5%	5%	6%	2%
Stage 3: Two leaves	7%	6%	7%	3%
Stage 4: Second node	3%	10%	0%	0%
Expected	**(+)**	**(+)**	**(+)**	**(-)**

Figure 12: Colonization of plants through the plant lifespan as expressed by average relative pathogen DNA in host samples, contrasted with the expected results. Error bars represent standard error.

Correlation to disease incidence at heading

Based on preliminary tests (*data not shown*), it was determined that the average success of inoculations was 55% infected heads per plant. Therefore, it was expected that the compatible reactions would be close to 55% successful at bunting ovaries. However, the success of inoculations at maturity was markedly lower than expected (*Figure 13*). While individual plants could be highly bunted, many plants were free of bunt which skewed the averages heavily. For example, individual cv. Yayla 305 plants infected with *T. laevis* race L-18 could be 100% sori out of up to 280 kernels. However, 80 out of 279 plants that developed kernels were completely uninfected (*data not shown*).

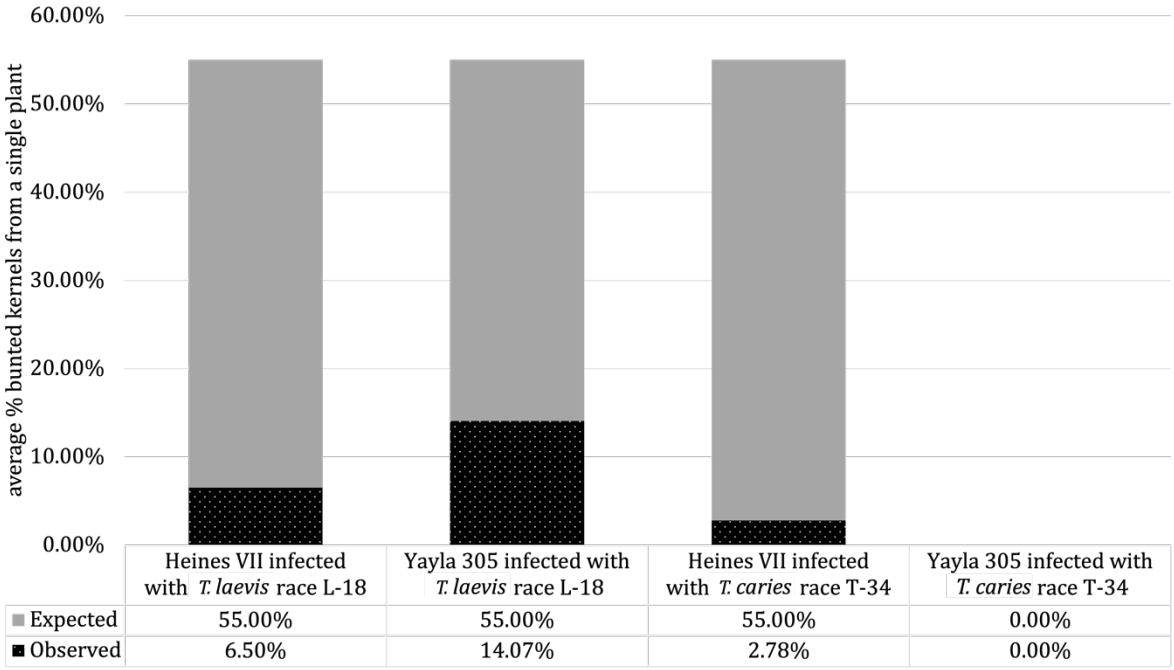


Figure 13: Bar chart of the expected vs. observed disease outcomes of each reaction between a cultivar and specific race of common bunt.

Non-destructively sampled first leaf samples generally showed higher relative pathogen DNA contents at the two-leaf stage and lower at the second node stage, although determining the impact of any specific tissues is hindered by the significant interaction of fungal race and growth stage in the model.

Separate Spearman correlation coefficients were calculated for first leaves and developing heads, the results are listed in *Table 2*. Correlations were consistently weak for all tissues analyzed.

Table 2: Spearman correlation coefficients for different tissues at different growth stages to disease incidence at heading, as rated by the average percent of sori out of all kernels

Growth Stage	Tissue	Spearman Correlation to Percent Bunted Kernels at Maturity
Two leaves	First leaf	0.61
Second node	First leaf	0.66
Second node	Developing head	0.67

Microscopy

The 0.01% TBO in PBS dye was able to differentially dye fungal cell walls and plant cell walls; the former dyeing magenta both in culture (Figure 14) and in plant tissue (Figure 15), while the latter dyed teal (Figure 15) to indigo (Figure 16; Figure 17) depending on the duration of the tissue's dyeing.

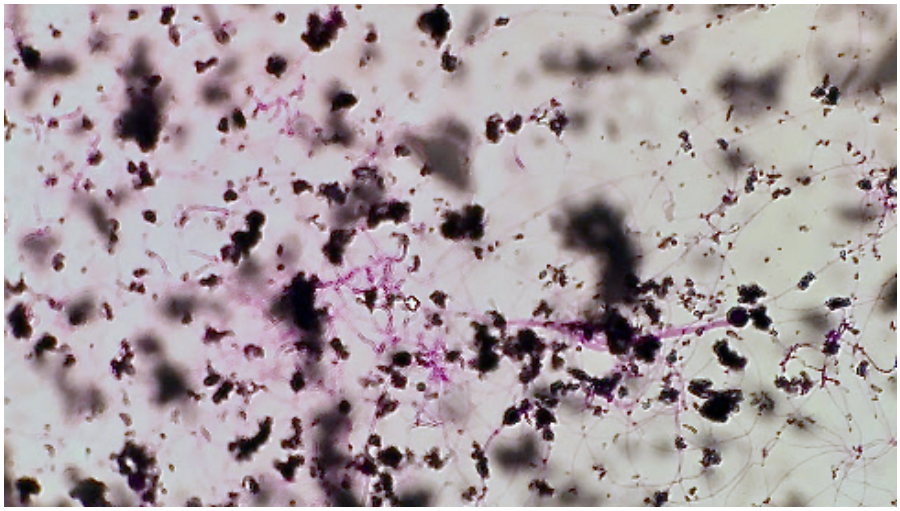


Figure 14: Image of cultured mycelium on water agar dyed with 0.01% Toluidine blue O in PBS (pH 7.4) and cropped to highlight hyphal threads. Hyphae are dyed magenta, while debris from sori exteriors remains black. Magnification 100X; photographed with iPhone 6S, cropped to highlight hyphae.

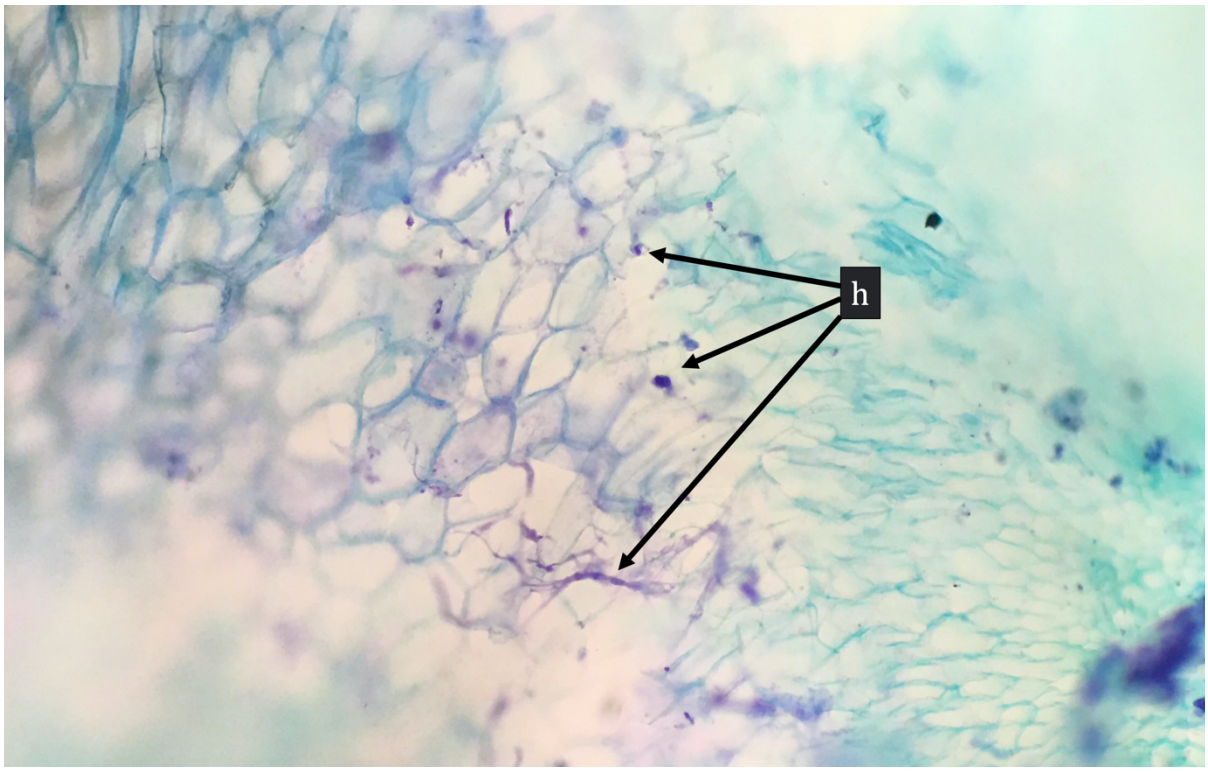


Figure 15: Infected crown from plant at Feekes stage 3, hand-sectioned and dyed with 0.01% Toluidine blue O in PBS, pH 7.4. Plant cell walls dye blue, and intracellular hyphal threads and cross-sections of hyphae (h; arrows) dye magenta. Magnification 400X; photographed with iPhone 6S, cropped to highlight hyphae.

The success of dyeing cross-sections of coleoptiles to determine infection or visualize hyphae was minimal. Hand-cut sections were often too thick to appropriately characterize. In initial work, the appropriate dyeing times for sections of different thicknesses was not determined. This resulted in over- (Figure 16a; Figure 17a, c) and under-dyeing (Figure 16b; Figure 17b, d) of different sections of differing thicknesses.

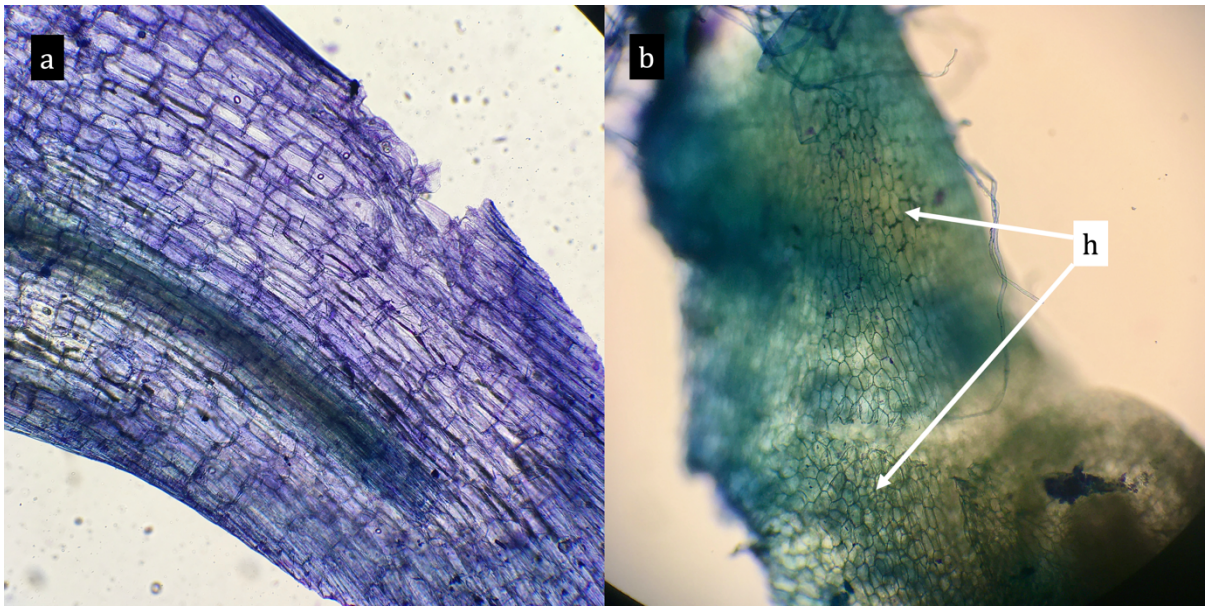


Figure 16: Images of coleoptile shoots hand-sectioned and dyed with Toluidine blue O in PBS, pH 7.4. Infection determined by qPCR of whole-coleoptile DNA extract. Magnification 100X; photographed with iPhone 6S, cropped to square. [a] Shoot from a pre-emergent 'Heines VII' (Bt0) coleoptile inoculated with Tilletia caries race T-34. The plant was allowed to absorb dye for longer than ideal, resulting in a higher uptake of dye and an exaggerated blue hue that may conceal intercellular hyphae in this section. [b] Shoot from a pre-emergent 'Heines VII' (Bt0) coleoptile inoculated with T. laevis race L-18. The thicker section was not allowed to absorb dye for the ideal amount of time, resulting in a lower uptake of dye but intercellular hyphae (h; arrows) seem to dye darker than the otherwise teal/undyed plant cell walls.

Some darkened cell walls were interpreted as hyphae (Figure 16b; Figure 17b), as they seemed to follow a path intercellularly that surrounded some cells. Not all darkened structures were likely hyphae, though. The under-dyed uninfected meristem in Figure 17d shows under-dyed areas that are translucent ivory in color, and amorphous blocks of colors ranging from teal to indigo, with cells below the focal plane appearing magenta. The lack of uniformity in dye uptake results in difficulty differentiating cells from each other, and the uneven cut of the section results in very narrow focal planes. The over-dyed sections (Figure 16a; Figure 17a, c) are heavily

dyed a dark indigo, and thicker over-dyed sections (Figure 17a) are blurred by out of focus pieces of the section.

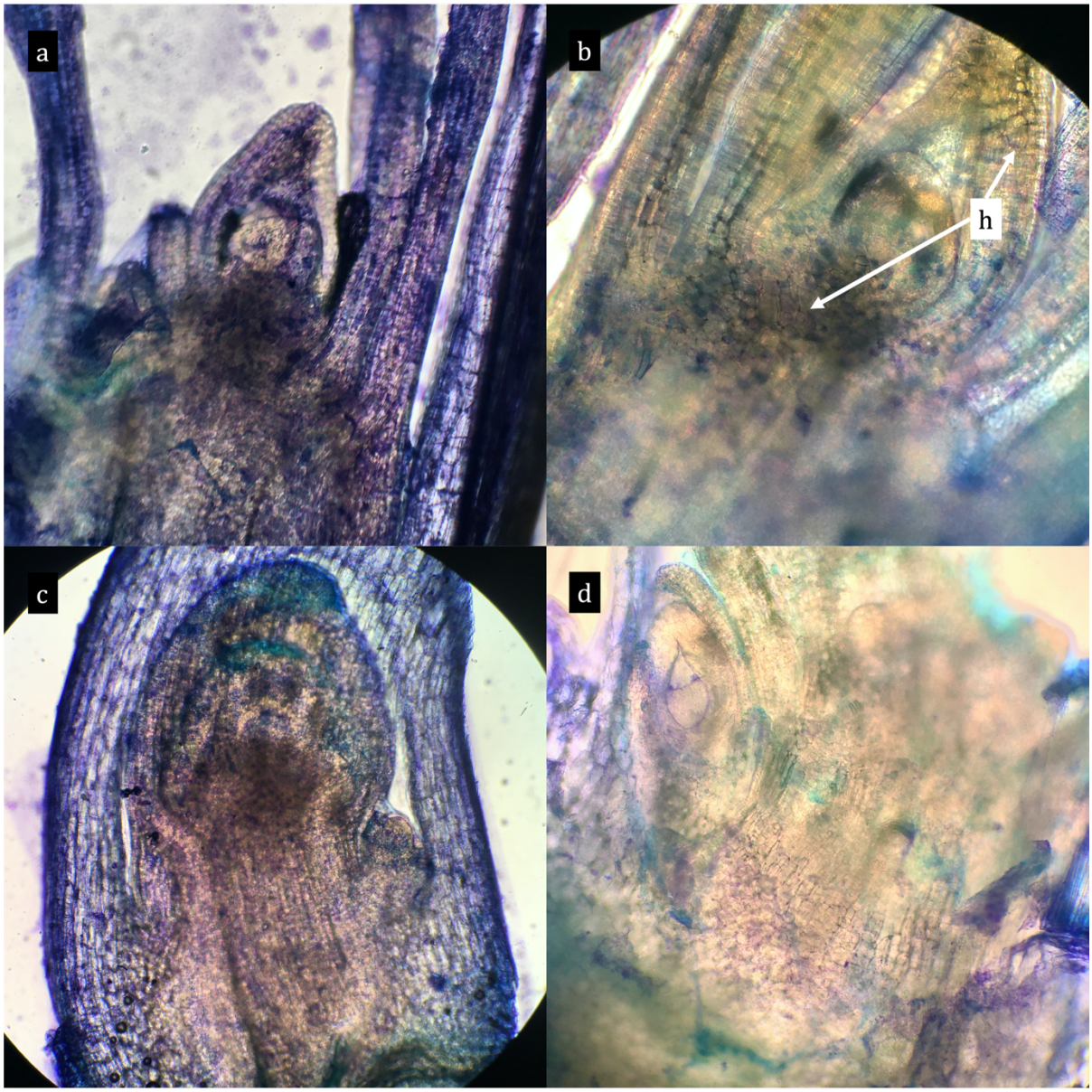


Figure 17: Images of coleoptile shoots hand-sectioned and dyed with Toluidine blue O in PBS, pH 7.4. Infection determined by qPCR of whole-coleoptile DNA extract. Magnification 400X; photographed with iPhone 6S, cropped to square. [a] Coleoptile meristem from a pre-emergent 'Heines VII' (Bt0) plant infected with Tilletia caries race T-34. The plant was allowed to absorb dye for longer than ideal, resulting in a higher uptake of dye and an exaggerated blue hue that may conceal intercellular hyphae in this section. It is possible

no hyphae were present in this section though, due to the fungus' variable growth habit in tissues; while the coleoptile may test positive not every cross-section will contain hyphae. [b] Coleoptile meristem from a pre-emergent 'Yayla 305' (Bt8) plant infected with T. laevis race L-18. The plant was under-dyed, though some magenta threads at the base of the meristem and in a developing leaf appear to be (h; hyphae. [c] Coleoptile meristem from a pre-emergent 'Heines VII' plant infected with T. caries race T-34. This plant was over-dyed as well, obscuring the ability to determine whether hyphae were or were not present in this section. [d] Uninfected meristem of a pre-emergent 'Yayla 305' plant inoculated with T. caries race T-34, showing the variability in colors in under-dyed tissue.

Discussion

This study aimed to improve existing methods for common bunt detection using qPCR and microscopy assays to allow for detection of common and dwarf bunt in plant tissue, with the goal of establishing the basis for a non-destructive early assay for screening germplasm for resistance in breeding. The development of an assay to measure relative fungal pathogen DNA content in host samples is novel in the use of endogenous controls for relative quantification of pathogen DNA in host samples, and that it requires much less specialized training to identify infection in any plant tissue before maturity and may avoid subjectivity associated with visual disease evaluation. The assay is sensitive to 0.1 pg of pathogen DNA, making it a highly useful tool in the study and diagnosis of common and dwarf bunt. The assay, also, requires no technical training beyond standard qPCR practices.

Table 2 demonstrates the attempt to correlate relative pathogen DNA in non-destructive leaf samples and developing heads samples to eventual disease outcome. No repeatable correlation was observed among the averages of the sample relative pathogen DNA content in a treatment group at a given growth stage with the average percent bunt balls in a plant in that treatment group. Neither first-leaf samples taken at the two-leaf stage nor at the second node stage, nor developing heads taken at the second node stage were predictive of disease outcomes. This indicates that although spread throughout the plant may be more abundant than previously thought, that

spread may not be sufficient to cause disease. However, infection outcomes were much lower than expected. More successful inoculations could have provided better insights into the relationships between amounts of pathogen DNA and disease incidence by providing a wider range of results. If a correlation were established, the significant differences between treatments at the second node stage indicates that this might be an appropriate time to perform a qPCR assay.

Zgraja et al. (2016) developed the qPCR assay to detect common and dwarf bunt in seed washes, which was useful for determining infection in seed lots of wheat, barley, and spelt. In their testing, their primers were able to detect only above 40 spores per seed. Though the exact conversion is unclear, this seems to have converted to a highly sensitive assay for detecting pathogen DNA in plant tissue. Theoretically the ability for the primers to detect 0.1 pg of pathogen DNA in a 20 ng standard amount of DNA allows for the detection of 1 molecule in 200,000 molecules. The methodology of this paper does not allow for conversion of that ratio to a mass ratio of fungal hyphae to wheat tissue, but the relative quantities suggest that a large portion of DNA extract from a tissue sample may be pathogen DNA. Detecting a range of relative pathogen DNA from seemingly the upper limit of probability (60.00%) to the lowest positive value of 0.04% suggests that the assay is sensitive to a wide range of colonization levels and is useful for distinguishing the extent of colonization. Further testing is necessary, though, to determine whether the assay is sensitive to DNA from dead hyphae as well.

Other researchers have looked to different tissues to characterize common bunt infection. Some have used the “third shoot” for their work, using qPCR, dot blot hybridization, and microscopy to cross-validate each other in the development of diagnostic tools (Kochanová et al. 2006). Eibel et al. (2005) collected roots, shoots and leaves, and compared results between ELISA and standard PCR. Others looked exclusively at apical meristems from the beginning of tillering, using a SYBR Green qPCR method to quantify the amount mycelial mass within the sample (Zouhar et al. 2010). Still others have used inflorescence tissue gathered at stem elongation, correlating disease diagnosis by their PCR assay to disease symptoms at maturity (Josefsen and Christiansen 2002). In this study, the use of first leaves did provide roughly equal correlation to eventual disease outcomes as did developing heads. This

contrasts with the findings of Josefsen and Christiansen (2002) that developing heads were a good predictive tool. Although this was proposed in 2002, and is corroborated to some degree here, diagnostics based on tissues at the stem elongation stage have not been adopted. This may be due to the laboriousness of sampling at this late stage, contrasted with the temporal proximity to heading at that time. In any case, none of these tissues studied have resulted in widespread shifts in diagnostics (Forster et al. 2021). Possibly, this is due to the destructive nature of sampling any of these tissues which precludes direct comparison to disease outcome and requires a larger planting to establish a higher resolution result.

While the similarity in different treatment groups at the earliest growth stages poses a problem for the utility of such sampling in a diagnostic sense for breeding, these results are congruent with studies that have been undertaken previously in which the initial growth stages of both resistant and susceptible wheat cultivars are initially infected in the same manner (Gaudet et al. 2007; Hansen 1958; Woolman 1930). This is interesting from a pathology standpoint, to interpret the action of resistance, but renders the earlier growth stages irrelevant to the outcome of disease and therefore inadvisable to correlate to disease incidence. The earliest difference in colonization between resistant and susceptible cultivars known to the author seems to occur sometime between the three leaf and two tiller stage (main stem and an additional tiller) (Xu et al. 2021). Further work will be done to determine the earliest common induction of resistance to better develop the earliest sampling protocol.

Some findings were unusual and are worth discussion. The averages for relative pathogen DNA were very low for most tissues in any of the treatment groups at most growth stages. The eventual disease outcomes also showed very low average infection, placing the compatible reactions firmly in the “resistant” territory of criteria set forth in 2021 (Madenova et al. 2021). This makes the work much harder to interpret, but to see any infection in those conditions, especially as widespread in the initial growth stages, suggests that the pathogens are more aggressive than asserted prior to this decade. It also seems as though the low averages are based on a lack of inoculation success, rather than a high ability for the cultivars chosen to resist the common bunt races chosen. Individual plants, when infected at all, were generally highly infected and these are

well-characterized reactions that have routinely shown a high level of disease (Goates 2012). It is possible that the pathogen was initially established very well, but methodology adopted later in the plant life cycle supported the plants in excluding the cold-weather common bunt. The author followed environmental control protocols as closely as possible but found a lack of information on the exact method of increasing the temperature between vernalization and maximum summer conditions. Tissue sampling was all done prior to or immediately after vernalization, but plants rated at maturity endured rapid heating to 27°C for the first trial replication and to 21°C for the second and third. It is thus speculated that much of the common bunt hyphae may have died when the plants were heat-shocked, making the results more complicated to interpret beyond that point. It is already well established that disease outcomes in any pathosystem depend on a favorable environment, and a lack thereof creates more difficulty in correlating early infection with disease outcome. It is possible, though, that a more complex model would need to be developed to factor in environmental parameters when associating infection at different stages of the life cycle.

Additionally, sampling protocols may have introduced some variability in the relative amounts of pathogen DNA calculated between tissue types. Pre-emergent coleoptiles were much smaller than emerging coleoptiles, but both were sampled the same way. It is possible that the general decrease in relative pathogen DNA in the second growth stage was due to a dilution effect caused by a similar amount of fungal mass between the second and first growth stages, but a much larger amount of wheat mass in the second growth stage. In future studies, this should be tested.

It is worth discussing, as well, the apparent resistance of Heines VII, the universally susceptible cultivar, to the common bunt race with the broadest known virulence spectrum to date, T-34. The discussion of heat stress to the plant could account for the low disease incidence at maturity in the Heines VII plants inoculated with T-34. That does not explain the lack of pathogen DNA in plants at the second node stage as compared to the other compatible reactions, though. One possible explanation would be the non-viability of the T-34 teliospores used. This was tested after the results were recorded, to determine if the spores had at some point become unviable between initial tests in 2019 and planting in December 2020. In March 2022, the teliospores

from that collection still germinated 100% on water agar, forming primary and secondary sporidia as well as H-bodies. This suggests that the spores were still highly infectious at the time of planting. It could be, though, that L-18 and T-34 need slightly different environmental conditions and that those used in this study favored L-18 over T-34 (Gaudet and Puchalski 1990).

Attempts to visualize infection in pre-emergent coleoptiles had very limited success. In the time in which this project was completed, the techniques for sectioning and dyeing were not fully developed. That stated, it was possible to use a polychromatic dye to differentially dye hyphae and plant cell walls differently in one single application of dye. Many microscopy protocols for the study of phytopathogenic fungi are laborious, multi-step processes that require clearing, fixing, embedding, microtome sectioning, dyeing and potentially counter-staining. These multi-step processes can be inaccessible for laboratories lacking advanced microscopy equipment and are more labor-intensive than the simpler technique presented here. Both more laborious processes and simple hand-sectioned processes require a fair amount of training and practice, though, to gather high-quality observations and images. While images were obtained, the sectioning procedure used resulted in thick sections that were not amenable to high-quality observations. The variability in section thickness, as a product both of inexperience and the challenging nature of the work, reduced the ability to standardize dyeing times which resulted in variability in dyeing success.

Of what was observed, it appears that hyphae likely spread intercellularly at the pre-emergence stage but that sections may not always reflect the infection status of a sample. Woolman (1930) noted that a "wide margin should be allowed for experimental error in examination; that is, a failure to find a few bits of small faintly stained mycelium in the great mass of tissue searched." The fungus colonizes many of the studied tissues, but at occasionally very low levels that may or may not be consistently spread throughout the sample. For example, Figure 16a and Figure 17c are the shoot and meristem, respectively, of the same coleoptile. The coleoptile had been divided in half, and half extracted of its DNA which was then tested by the qPCR method, returning a relative pathogen DNA content of 28.9%. Although this suggests that a large amount of hyphae might be seen in every section, errors in dyeing and sectioning as well as

uneven distribution of hyphae within the coleoptile may have contributed to a lack of visual confirmation of the qPCR results.

To conclude the discussion of this work, this project used existing primers and technology to develop a qPCR assay that is capable of detecting trace amounts of common bunt DNA in small wheat samples. In utilizing this assay, it was found that some reactions show some promise for developing a non-destructive assay, but it seems unlikely to be a generalizable linear relationship and the low disease pressure makes it hard to state confidently at this time. Given that the exact timing of the resistance action was not discerned in this study, marker-assisted selection of known effective genes (MAS) may be a more appropriate tool for breeders than earlier non-destructive qPCR diagnostics. As markers continue to be developed and validated for effective genes against bunt, they may shorten the breeding timeline more effectively while this assay may contribute to disease pathology research more effectively. Given the similarity in genetics and virulence and avirulence factors between common and dwarf bunt, this assay is likely appropriate for use in those systems as well.

As more research is done on the subject, the results of this study suggest that the highly genotype-specialized course of infection and the different results given in different reactions at different times necessitate an explicit statement of these parameters in any future methods sections to preserve reproducibility and comparison between studies. The cultivar selected must be named and its resistance genotype described, if known. Common bunt isolates must be listed, and if unknown the lack of information must be stated. The timing of sampling, the environmental conditions, and the type of tissue must all be carefully reported as all these factors may contribute to the observation of relative pathogen DNA content.

Future work should test different conditions to determine the most conducive conditions for infection of these cultivars to maximize infection for better correlation potential. Microscopy techniques should be standardized, and observations and images taken with higher-quality cameras, microscope software, and photo editing software. The assay should be developed and validated across multiple isolates and host genotypes, in different labs, and additionally with more financially accessible reagents. As new primers are developed that can discern common bunt fungi from dwarf bunt

fungus, these should be incorporated into the assay. The current results should be interpreted cautiously, given that previous studies have been criticized for a lack of isolates in study (Bao 2010). This study utilized only two isolates, *T. laevis* race L-18 and *T. caries* race T-34, which was sufficient to determine a preliminary idea of how qPCR may or may not be used for breeding purposes. However, if used for early diagnostics this assay should be validated among the entire set of resistant differentials and significantly more isolates. Although ITS differences in pathogen races are highly unlikely due to the genetic stability of the ITS region between species of common bunt and dwarf bunt, the variability in colonization modes between and within compatible and incompatible reactions suggests that in certain genotypes early positives may not be indicative of eventual disease.

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Chapter 3:

Major bunt resistance gene *Bt8* interactions allow for differing levels of tolerance to common bunt (*Tilletia laevis*, *T. caries*) in wheat (*Triticum aestivum*)

Abstract

Common bunt, caused by both *Tilletia laevis* and *T. caries*, is a reemerging disease of wheat with the potential to devastate unprotected crops globally. Breeding for host resistance has been the control method of choice for over 100 years, utilizing one or more of 16 known major resistance genes in a given region in race-specific reactions. However, assessing host resistance classically requires completing the entire plant life cycle and takes months. Little has been published on the progress of colonization in incompatible reactions, and even less so on the manner in which specific resistance genes influence fungal growth. Research on the often-deployed resistance gene *Bt10* established a rapid interaction between host and pathogen that results in the death of the common bunt fungus. It was unclear whether *Bt8*, the most important resistance gene used in the Pacific Northwest, would provide the same level of immediate control. An experiment was designed using a newly modified qPCR assay to trace the fungus in compatible reactions in both the universal suscept, *Triticum aestivum* cv. 'Heines VII,' and the *Bt8* differential cv. 'Yayla 305' using *T. caries* race L-18 and the incompatible reaction between 'Yayla 305' and *T. caries* race T-34 by sampling at 4 different growth stages throughout the plant life cycle. Additionally, non-destructive leaf samples were taken to attempt a correlation between the relative quantity of pathogen DNA in these samples and eventual disease expression (percent sori production) in order to develop an assay to shorten the timeframe for determining resistance in breeding populations. Due to a lack of consistent types of fungal colonization between compatible reactions of different host genotypes, and a markedly different resistance timeline development between *Bt8* and *Bt10*, this study concludes that not only is resistance race-specific, it is also host-genotype dependent as is compatibility. This necessitates a higher level of

genotype reporting for future studies on the subject, and more research into the general timing of resistance in incompatible reactions if a qPCR assay is to be utilized.

Introduction

Wheat, one of the world's staple crops, is threatened by the reemergence of common bunt in unprotected volunteer wheat and systems in which chemical seed protectants cannot either be applied or afforded. Without protection, the causal fungi (*Tilletia caries* (A.P. de Candolle) L.-R. & C. Tulasne (syn. *T. tritici* (Bjerk.) G. Winter), and *T. laevis* J.G. Kühn (syn. *T. foetida* (Wallr.) Liro)) can replace the developing kernel with a lookalike sorus (bunt ball), that breaks open during harvest to release millions of fetid spores. Contamination of seed lots with common bunt spores results in a reduction in value or inability to sell the seed lot, and due to the difficulty in discerning common bunt spores from the quarantine pathogen dwarf bunt (*Tilletia controversa* Kühn), may result in rejection or destruction of exported grain. Breeding resistant varieties has been the most effective method of control which is costly in terms of the time required for screening and incorporation of resistance but could be accelerated with access to new technology. Resistance remains a breeding target as new susceptible varieties are introduced to areas of the Pacific Northwest where common and dwarf bunt are endemic and as the pathogen mutates and hybridizes, which requires a better understanding of the mechanisms of resistance in winter wheat.

Previous research of common bunt colonization in resistant cultivars determined the exclusion of the pathogen corresponded to the deposition of callose around the infection point of the fungi (Gaudet et al. 2007; Woolman 1930). Other research reported that some incompatible reactions more closely resemble a tolerant quantitative reaction than a qualitative resistant one. In Woolman's 1930 histopathology study, although he concluded that resistance was rather swift, beginning with fungal suppression at about 9 days and almost entirely preventing colonization beyond the coleoptile and leaf sheaths by 20 days, one table in the paper reported that a somewhat resistant cultivar was still harboring fungal hyphae at 60 days post-inoculation. This may indicate that some compatible reactions more closely resemble a tolerant quantitative reaction than a qualitative resistant one. A study by Griffith (1953)

found mycelia in the otherwise healthy kernels of plants that did not develop disease. While these earlier studies hinted at a more complicated interaction between host and pathogen, in Gaudet et al.'s 2007 study reporting on the *Bt10* resistance gene as challenged by *T. caries* races T-1 and T-27, they determined that although infection events can occur from germinating spores up to 21 days post-inoculation, in incompatible reactions each infection event receives a rapid defense response and is prevented from further colonizing additional tissue. They concluded that this was evidence for a lack of systemic resistance responses to common bunt fungi, stating that "the pathogen could never maintain the growth rate necessary to attain even low levels of infection and proliferate in the developing spike, if it was subjected to a continuous defense response." In regard to the common phenomenon of low (<10%) infection rates occurring in resistant varieties, they pointed to environmental moderation of resistance genes and the resultant breakdown of resistance in unfavorable conditions as more likely.

Despite their inferences about a lack of systemic response, Gaudet et al. (2007) did note that further study on the subject would be required to deepen the scientific understanding of the host-pathogen relationship. Different resistance genes are more effective in different geographical regions as the population structure of fungal races shifts with location. In the United States Pacific Northwest, *Bt8* has been one of the most successful and commonly used resistance genes (Goates 2012, 1998; Waud and Metzger 1970). To further develop our understanding of common bunt colonization in different host genotypes, and to protect the longevity of *Bt8* effectiveness, this research encompassed a whole-host-lifespan qPCR-based approach to track the fungi through compatible and incompatible *Bt8* reactions in comparison to fully compatible reactions in the absence of resistance genes (*Bt0* genotype). Given that common bunt and dwarf bunt are closely related, it is likely that these results extend to wheat interactions with dwarf bunt (Goates 1998).

The objectives of this study were to (i) use a newly modified multiplex qPCR assay to trace the spread of the fungi through five different host growth stages, (ii) determine the differences in colonization and defense induction between compatible and incompatible reactions involving two different host and pathogen genotypes each

at each studied growth stage, and (iii) attempt a correlation between infection in at least one studied growth stage with classically-rated disease incidence at maturity in order to (iv) develop a non-destructive diagnostic assay for early detection of common and dwarf bunt resistance in wheat.

Materials and Methods

Experimental Design

Three replications of this experiment were conducted in controlled environment chambers from December 2019-July 2021. To study the difference between compatible and incompatible reactions, in each replication two cultivars with different susceptibility patterns were challenged with each of two races of common bunt. Specifically, *Triticum aestivum* subsp. *aestivum* cv. 'Heines VII' (carrying *Bt0,* with no known resistance) and *T. aestivum* subsp. *aestivum* cv. PI 178210 'Yayla 305' (carrying *Bt8,* a source of broad-spectrum and durable resistance gene) were both challenged with *T. laevis* race L-18 and *T. caries* race T-34. Of these, only the combination of 'Yayla 305' and *T. caries* race T-34 results in an incompatible reaction, showing less than 7% common bunt infection at heading (Matanguihan and Jones 2011).

Given the goal of the study to describe the growth of the common bunt fungus throughout the wheat life cycle, five growth stages were selected for study;

6. Pre-emergence – Zadoks 5, Feekes 0
7. Emergence – Zadoks 7-9, Feekes 1
8. 2nd leaf – Zadoks 12, Feekes 1
9. 2nd node detectable – Zadoks 32-39, Feekes 7
10. Maturity – Zadoks 85-92, Feekes 11.2-11.3

The first four growth stages were sampled for DNA extraction and analysis with qPCR. The sixth growth stage was rated for disease phenotype (as percent of bunted kernels out of all kernels on a plant) at maturity.

Each of the three replications of the experimental trial consisted of at least 54 plants per combination of cultivar and race, of which 5-9 plants of each were sampled at each growth stage along with 2-3 plants of each control group at each growth stage. The number of plants sampled was dependent on germination of seed and time constraints.

Seed and Inocula Source

Wheat (*Triticum aestivum*) seed of two varieties were obtained from the National Small Grains Collection (NSGC) in Aberdeen, ID. PI 178210 (cv. 'Yayla 305'), a pureline winter wheat bred from a Turkish landrace was used as the *Bt8* differential. The *Bt0* differential cv. 'Heines VII', a hard red winter wheat, was used as the universal suscept. Seed of both cultivars were increased in 2019 and 2020 by growing out plants in the Conviron growth chambers (Conviron, Pembina, ND, USA) and greenhouse facilities at the University of Idaho Research and Extension Center in Aberdeen, ID.

Previously identified and collected isolates of *T. laevis* race L-18 and *T. caries* race T-34 were used for inoculations. L-18 sori were sourced from the collections of Dr. Jianli Chen (University of Idaho wheat breeding program), which had been grown on the susceptible wheat cultivar 'Rio Blanco'. T-34 sori were sourced from the original collection of Dr. Janet Matanguihan in Pullman, Washington.

Culture Preparation

T. laevis and *T. caries* isolates were surface sterilized and incubated on 2% water agar (Difco® Bacto® Agar, Becton, Dickinson and Company, Sparks, MD) in the dark at 15°C for 3-5 days. Hyphae were transferred to 3.9% potato dextrose agar (Becton, Dickinson and Company, Sparks, MD) and cultured under the same conditions for at least 2 weeks for use as fungal DNA standards after DNA extraction.

Inocula Preparation

Sori of isolates *T. caries* race T-34 and *T. laevis* race L-18 were stored dry in a sealed sterile tube at room temperature until use. Sori were sterilized in 5% household bleach for 20 seconds, rinsed 3 times in sterile water for 20 seconds each rinse, and dried gently with a sterilized Kimwipe. 4 sori were crushed and suspended in 750 µl of 0.5% methyl cellulose solution (Carolina Biological Supply Company, Burlington, North Carolina) for each lot of 100 seeds.

Inoculation

Inoculation followed a modified protocol described by Goates (2012), utilizing the preparation of an aqueous preparation of 0.5% methyl cellulose (Carolina Biological Supply Company, Burlington, North Carolina).

Seeds were inoculated with the surface sterilized spores in methyl cellulose solution without prior seed surface sterilization. Seeds were allowed to soak in the inoculum solution for 1.5-2 hours at room temperature in light conditions. Seeds were then placed 5 cm. deep in either a square 4-inch pot (100% of plants for Trial 1 and 50% of plants for Trials 2 and 3) or white Ray Leach Low Density Cone-tainer™ (Steuwe & Sons, Inc., Tangent, OR) (50% of plants in Trials 2 and 3) filled with a moistened blend of 40 parts peat moss, 40 parts sand, 40 parts vermiculite and 1 part Osmocote™ Outdoor and Indoor Plant Food fertilizer. Seeds were germinated in a Conviron growth chamber set to 12 hour 10 ℃ full light periods with 5 ℃ full dark periods.

For each trial, a set of mock inoculated seeds of each variety was also included as a negative control. Mock inoculation consisted of vortexing and soaking the seed in the methyl cellulose solution for the same duration as the seeds inoculated with the teliospores. They were then grown in the same conditions and sampled in the same way at the same times as the teliospore-inoculated plants.

Growth Conditions

After growing past Feekes 1 (Zadoks 12), the plants were vernalized for 8 weeks with 8 hours of full light at 8 ℃ and 16 hours of full dark at 5 ℃. At the end of 8 weeks, the temperatures were gradually raised for optimum plant growth. For Trial 1, this was done by increasing the temperature by 3°C weekly up to a final temperature of 27°C. For Trials 2 and 3, after observing heat stress in Trial 1, conditions were gradually warmed to a 16-hour full light period at 21°C, with an 8-hour dark period at 10°C. Plants were watered with tap water (hardness >180 mg/liter) on a regular schedule. No additional fertilizer was applied.

Sampling

Sampling was done using sterilized equipment and surfaces at the University of Idaho Aberdeen Research & Extension Center. Each growth stage was sampled using techniques best fitting for the plant tissues at that stage. After sampling, samples were preserved at -20 °C for up to 2 weeks and then -80 °C until extraction.

For pre-emergent seedlings (Zadoks 5, Feekes 0, *Figure 1*), plants were gently removed from the Cone-tainer soil and if necessary, briefly and carefully washed in still Millipore water to preserve, as much as possible, the fungal mass on the coleoptile. Seedlings were then removed from the seed and divided into a root sample and a coleoptile sample. The root sample was preserved in a clean microcentrifuge tube. The coleoptile was bisected longitudinally, with one half preserved in a clean microcentrifuge tube for freezing and eventual DNA extraction. Seedlings at emergence (Zadoks 11, Feekes 1, *Figure 2*) were sampled much the same as pre-emergent seedlings.

Figure 18: A pre-emergent seedling, representing the first sampling growth stage.

Figure 19: An emerging seedling, representing the second sampling growth stage.

Seedlings at the 2-leaf stage (*Figure 3*) were gently removed from the Cone-tainer soil, and the roots cut with clean scissors 3 cm. below the seed. The remaining soil was carefully scraped away, and the roots were then swirled in still Millipore water

until clean. The seed coat was then discarded, and roots were cut off from the plant and frozen. After this, the crown along with the 1 cm. of developing tissue surrounding the crown was cut from the stem. The crown was bisected longitudinally, and one half frozen. Left with the stem, consisting of the first leaf and coleoptilar sheath surrounding the second leaf, the leaves were cut away 3 cm. above the point at which they diverged from each other (where the second leaf fully emerges). The first leaf was then gently pulled away from the second, bisected longitudinally, and one half frozen. The second leaf was then bisected longitudinally, and one half frozen.

Figure 20: A plant at the two-leaf stage, representing the third growth stage.

Plants at the older growth stage, Zadoks 32-39 (Feekes 7 (*Figure 5*)), were removed from the soil and their roots cut away with sterilized scissors. The crown, often tough and woody at this stage, was cut from the tiller bases and bisected. If the crown was larger than 6 mm^3, it was cut into smaller portions representatively and one half frozen. The three most developed tillers were then selected from the remaining plant. For each tiller, the first leaf was pulled away from the tiller, cut to roughly 7 cm. from the base, bisected longitudinally, and one half frozen. The remaining stem was then dissected. The first node was removed, bisected longitudinally, and one half frozen. The second node was then dissected the same. At this point, tillers were developmentally different from each other and thus the entire stem was bisected longitudinally to better visualize the developing tissues inside the stem. Third nodes, and if present fourth nodes and developing heads were cut away from the stem and one half frozen.

Figure 21: A plant at the second node stage, representing the fourth growth stage.

Rating Percent Disease Incidence

Classical disease rating has involved visually assessing the proportion of bunted heads in a stand and relying on necessarily subjective cutoffs to determine resistance or susceptibility. As discussed in the literature review chapter of this thesis, this is an unsatisfactory approximation of the range of resistance responses in wheat. To add precision to this measurement, percent disease incidence (% DI) was recorded as both per treatment % DI (infected plants out of the total stand) and per plant % DI (sori out of all developed ovaries).

Percent disease incidence of a plant was used to refer to the proportion of sori (infected kernels) out of all developed "kernels" present on a plant. Plants were scored for number of infected heads out of the total number of heads. After this initial rating, individual spikelets were opened to tally the number of seeds and number of sori. Disease incidence (% DI) for each plant was then calculated as:

$$\%DI = \frac{s}{\sum s + k}$$

Equation 5

where s is the number of sori total across spikes and k the number of healthy kernels across spikes.

Percent disease incidence per treatment was calculated as the ratio of diseased plants (plants with at least 1 sorus, p_d) out of all plants in the treatment (p_t):

$$\%DI = \frac{p_d}{p_t}$$

Equation 6

DNA Extractions

DNA was extracted from both frozen mycelia and healthy wheat tissue for fungal and host DNA standards, respectively. Total genomic DNA was also extracted from frozen halved inoculated host tissues using the Norgen Biotek Plant/Fungi DNA Isolation Kit (Cat. 26250, Norgen Biotek Corp., Thorold, ON, Canada). Sample cells were disrupted

using a Spex Sample Prep Geno/Grinder (Spex SamplePrep, Metuchen, NJ) and two 2 mm. steel beads. The samples were ground to a paste in their tubes, using the GenoGrinder set to 1500 rpm for 1-5 minutes as necessary, with sets of 5 minutes repeated as necessary for older plant tissue. Ground samples were then frozen again for at least one day. After this, the Norgen kit was used as directed.

Pure cultured mycelium DNA was extracted after freezing at -80°C for at least 1 week. Mycelium cells were disrupted by pulverizing thawed tissue in 600 µl Lysis Buffer (Norgen Biotek Plant/Fungi DNA Isolation Kit, Cat. 26250, Norgen Biotek Corp., Thorold, ON, Canada) with 2 mm steel beads on the Spex Sample Prep Geno/Grinder. The suspended tissue was shaken at 1500 rpm for 5 minutes, until the cells were evenly distributed in the Lysis Buffer. After cell disruption, the Norgen kit was used as directed.

DNA Dilutions

DNA concentration and quality were read on a BioTek Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Samples were then diluted with 1X Tris to either 20 ng/µl, 10 ng/µl, or 5 ng/µl depending on the concentration of the extract, and stored at -20°C.

Preliminary Tests

Primers developed by Zgraja et al. (2016) (see **Error! Reference source not found.**) w ere tested with SYBR Green and BioRad Precision Melt Supermix with a final melt curve to assess PCR products for dimerization. Each well contained 10 µl Precision Melt Supermix, 1 µl of each 2 µM primer, 6 µl molecular biology grade water, and 2 µl DNA template totaling 20 ng genomic DNA of pure cultured L-18 mycelium. Cycling conditions were a 2-minute hold at 95 °C, then 40 repeats of 95 °C for 10 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds followed by a plate read. After 40 repeats, the melt curve analysis was developed by increasing the temperature from 70 °C to 90 °C in 2 °C increments, with a plate read every 5 seconds. This produced both a series of fluorescence reads (curves) and a melt curve. Fluorescence curves were visually assessed for Ct value stability, curvature, and strength of amplification. The melt curve

was visually assessed for the solitude of the peak. These being satisfactory, the Zgraja et al. (2016) primer-probe combination was tested in a multiplex qPCR and worked well.

Primers designed to amplify common reference genes with stable expression in wheat selected from the work of Wei et al. (2015) (see *Table **3***) and were tested by a SYBR Green BioRad Precision Melt Supermix in both a non-template molecular grade biology water control and a series of samples representing different tissues and growth stages from this project. Each 20 µl reaction volume consisted of 10 µl Precision Melt Supermix, 1 µl of each 2 µM primer being tested in that well, 6 µl molecular biology grade water, and 2 µl DNA template totaling 20 ng genomic DNA. Cycling conditions consisted of a 2-minute hold at 95 °C, then 40 repeats of 95 °C for 10 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. After the 40th repeat, the plate was held at 4 °C. This produced a series of fluorescence curve reads, which were visually assessed for variation in Ct values across sample types and strength of amplification. The PCR products were then run in duplicate on a 1.5% agarose gel at 90 volts for 1.5 hours to verify the size of the amplicon and the number of PCR products.

After selecting the AB181991 primers published by Wei et al. (2015), it became necessary to design a probe. The probe for this primer pair was designed using Primer3Plus (Untergasser et al. 2012) with the source sequence from the National Center for Biotechnology Information (NCBI), and flanking primer sequences from Wei et al. (2015). Default settings were used, other than an adjustment to the GC% to a minimum of 30, optimum of 40, and maximum of 50. The resulting sequence (*Table **3***) was used to develop the probe AB181991-Pr tagged with [HEX] and [BHQ1] (Eurofins Genomics). This probe was validated against DNA from multiple wheat tissue types and DNA from pure fungal cultures, and the concentration of the probe in the reaction was optimized for use in the qPCR conditions specific to the Universal Express qPCR SuperMix (Invitrogen).

Primers were consistently used in multiplex as 1 µl at a 10 µM concentration. This was selected given the standard practices of qPCR in plant pathology and confirmed through repeated use in multiplex conditions. Each 25 µl reaction well consisted of 12 µl Universal Express SuperMix (Invitrogen, Waltham, MA), 5 µl molecular biology grade water, 1 µl of each wheat as well as fungal primer (10 µM), 1 µl

of wheat probe (variable concentrations), 1 µl of fungal probe (variable concentrations), and 2 µl template DNA totaling 20 ng genomic DNA. Real-time PCR thermal cycling was conducted using Bio-Rad CFX96 (Bio-Rad Laboratories, Hercules, CA). Cycling conditions were described by the manufacturer for Universal Express SuperMix: 50 °C for 2:00 mins, 95 °C for 2:00 minutes, 60 °C for 1:00 minute followed by a plate read and 40 cycles of amplification then a hold at 4 °C. Given the repeated amplification in a consistent manner, this concentration was considered optimal and acceptable.

Probes were tested at three concentrations; 5 µM, 2 µM, and 1 µM. Multiplex conditions were continued as previously described, and the results were visually assessed for consistency of Ct values in different reactions of the same sample and amplification strength. The strongest and most consistent concentration was selected. In the case that two or more concentrations were comparable, the middling concentration was selected to balance material use and efficiency.

After confirming the performance of both the selected fungal primers and probes, and the wheat primers and probes in multiplex with each other, the primers and probe selected from Zgraja et al. (2016) and Wei et al. (2015)'s primers and the herein developed probe based on the AB181991 sequence were used for all qPCR reactions.

Limit of Detection

After optimizing concentrations of primers and probes, the limit of detectable DNA amounts was tested for each oligo set. A dilution series was produced consisting of 10 ng/µl, 5 ng/µl, 0.5 ng/µl, 0.005 ng/µl, 0.0005 ng/µl, and 0.00005 ng/µl. Each respective dilution solution was used at 2 µl to create a series of 20, 10, 1, 0.1, 0.01, and 0.001 ng genomic DNA in each respective well. Each 23 µl well contained 12 µl Universal Express SuperMix (Invitrogen, Waltham, MA), 8 µl molecular biology grade water, 1 µl of both 10 µM primers, 1 µl 2 µM probe, and 2 µl of the respective genomic DNA dilution. Cycling conditions were: 50°C for 2:00 mins, 95°C for 2:00 minutes, 60°C for 1:00 minute followed by a plate read and 50 cycles of amplification then a hold at 4°C. The default software settings were used to calculate the threshold and Ct values.

qPCR Conditions

Each sample was run through a multiplex PCR assay intended to amplify the host DNA and any present pathogen DNA. To achieve this, 2 sets of oligos were used – one specific to wheat with a HEX-labeled probe and the other specific to *T. caries*, *T. laevis*, and *T. controversa* with a FAM-labeled probe (Table 1). All samples were run in duplicate, with both positive (pure wheat DNA and, separately, pure *T. laevis* DNA) and negative (molecular biology-grade water) controls performed in duplicate with each plate. Primers (Table 1) were ordered from Eurofins Genomics (Louisville, KY, USA). Each 25 µl reaction well consisted of 12 µl Universal Express SuperMix (Invitrogen, Waltham, MA), 5 µl molecular biology grade water, 1 µl of each wheat as well as fungal primer (10 µM), 1 µl of wheat probe (2 µM), 1 µl of fungal probe (2 µM), and 2 µl template DNA totaling 20 ng genomic DNA. Real-time PCR thermal cycling was conducted using Bio-Rad CFX96 (Bio-Rad Laboratories, Hercules, CA). Cycling conditions were: 50°C for 2:00 mins, 95°C for 2:00 minutes, 60°C for 1:00 minute followed by a plate read and 40 cycles of amplification then a hold at 4°C. The default software settings were used to calculate the threshold and Ct values. Samples were considered positive for common bunt if the Ct values were lower than the average Ct value of the 0.1 pg sample, as limit of detection testing demonstrated that 0.1 pg was the lower limit of detection and negative controls were not amplified before that cutoff. This provided stability between plates.

Table 3: Sequences of primer-probe sets utilized to amplify fungal DNA from common bunt -infected wheat tissue

Name	Gene Accession #	Sequence	Amplicon length (bp)	Target	Source
AB181991	AB181991	**F:** AGCGGTCGAACAACTGGTA	101	wheat ACT	Wei et al. (2015)
		R: AAACGAAGGATAGCATGAGGAAGC		wheat ACT	Wei et al. (2015)
		P: [HEX]-TGAGCCACACTGTTCCAATC-[BHQ1]		wheat ACT	this work
Til122	HQ317580	**F**: ACCCATTGTCTTCGGACTTG	140	*Tilletia* spp. ITS region	Zgraja et al. (2016)
Till262		**R**: GGTGCGTTCAAAGATTCGAT		*Tilletia* spp. ITS region	Zgraja et al. (2016)
Til175		**P**: [FAM]-CTTGGTTCTCCCATCGATGAAGA-[BHQ1]		*Tilletia* spp. ITS region	Zgraja et al. (2016)

Note: F – forward primer, R – reverse primer, P – probe

Primer Efficiency Calculations

Efficiencies for each of the two primer sets used were calculated based on an average of the runs of the 10-fold dilution series performed. For each run of the dilution, the average Ct of each diluted sample was calculated and plotted against the log of the sample quantity. The slope of the regression line was calculated using Excel Version 16.59's "SLOPE" function, and the R^2 value using the program's "RSQ" function. The efficiency was then calculated using Equation 7;

$$E_{primer} = (10^{\frac{-1}{slope}} - 1) \times 100$$

Equation 7

The efficiencies were separately calculated for at least two runs of the standard curve, and then averaged for use in relative DNA quantification.

Quantification

Ct values were recorded for each well after each run and were averaged between the two duplicates to determine an average wheat host Ct (hCt) and an average bunt pathogen Ct (pCt). Controls of a known 0.001:1 mix of pure fungal pathogen DNA and pure wheat DNA were run seventeen times to determine an average control value for both hCt and pCt.

The Pfaffl equation (*Equation 4*) was then used to calculate the relative amount of bunt DNA in a sample (Pfaffl 2001).

$$ratio = \frac{(1 + E_{bunt})^{\overline{(pCt_{control}} - \overline{pCt_{sample})}}}{(1 + E_{wheat})^{\overline{(hCt_{control}} - \overline{hCt_{sample})}}}$$

Equation 8

Data Analysis

After calculating the relative amount of bunt DNA within host samples, analysis was done using SAS v. 9.4. Only data for inoculated groups was considered, as the controls demonstrated a lack of contamination. To analyze data that contained multiple 0 values due to unsuccessfully inoculations or incompatible reactions, a value of 0.00000001 was added to all relative pathogen DNA values. Data was sorted according to trial replication, race of fungal inoculum, cultivar, and growth stages 1-4. PROC MEANS was then employed to create a table of mean relative pathogen DNA values for each replication/race/cultivar/growth stage group. This table of means was then modeled using PROC GLIMMIX to log transform the data and develop a generalized linear mixed model with a random complete block design. The random effect was set as the interaction between trial replication, bunt race, wheat cultivar, and growth stage. The fixed effects were cultivar, bunt race, growth stage, and all combinations of the three factors. The outputs of this model were assessed for significance by studying the p-values and effect sizes of the overall model and parameters.

Spearman correlations between relative pathogen DNA and disease incidence at maturity were done using the PROC CORR function in SAS v. 9.4 with the “Spearman” option.

Results

Covariate analysis

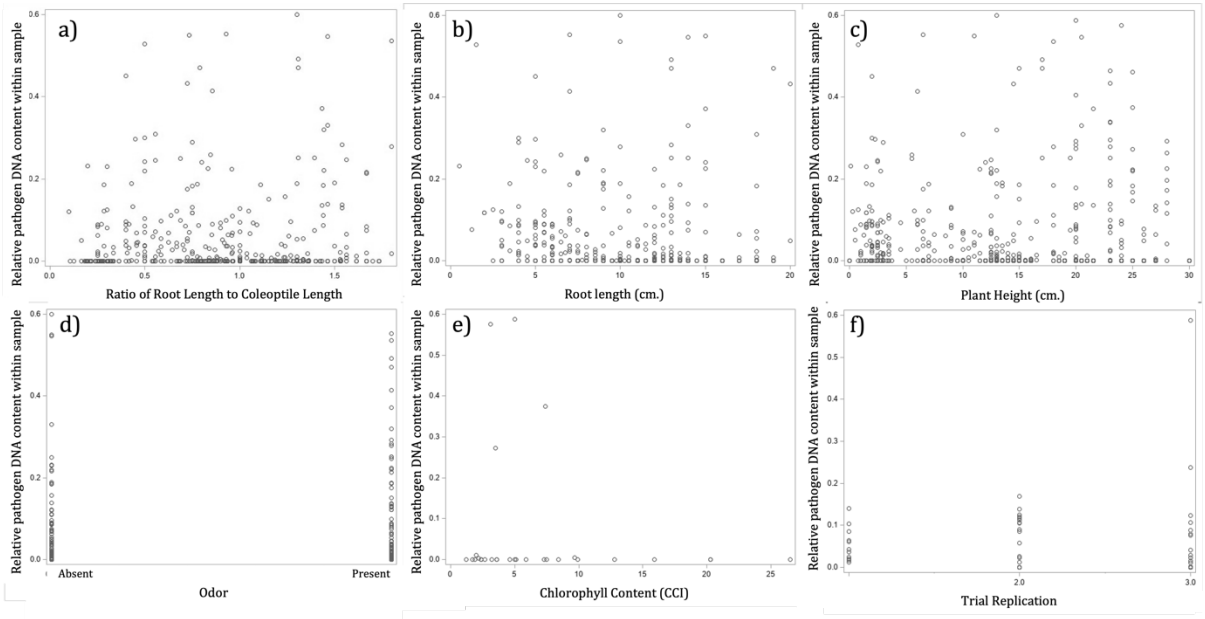


Figure 22: Scatter plots of potential covariates showing a lack of relationship. a) No relationship between root: shoot ratio and disease outcome. b) No relationship between root length and disease outcome. c) No relationship between plant height and disease outcome. d) No relationship between odor and disease outcome. e) No relationship between chlorophyll content and disease outcome. e) No relationship between trial replication and disease outcome.

Scatter plots were used to visually assess the need for further exploration of potential covariates (Figure 22). The ratios of root length to coleoptile length were randomly associated with the disease outcome of the same sample, as were the measurements of root length and coleoptile length/plant height. Samples were randomly associated with the presence or absence of the metallic, rotten odor. Chlorophyll content also seemed to have no correlation to disease outcome.

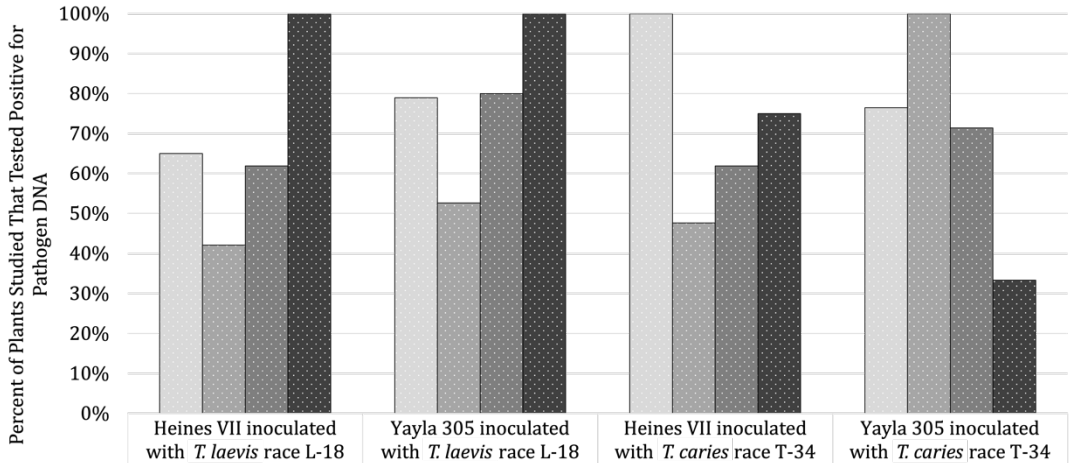
Trial outcome seemed as though there were a wider spread of relative pathogen DNA values in the third replicated trial, however, a one-way ANOVA of the relationship between trial replication and relative pathogen DNA content in a sample resulted in an

F-value of 0.22 and a p-value of 0.8018. The correlation, therefore, was determined to be insignificant.

Progression of pathogen colonization of host

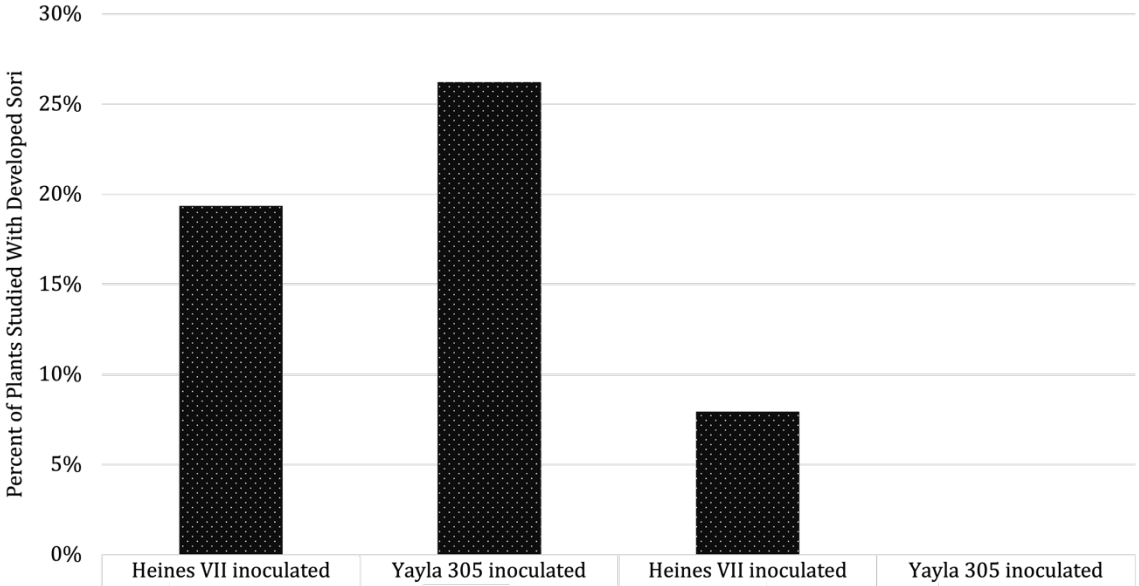
Observationally, there were no visual differences between compatible and incompatible reactions until heading. All tissues looked as expected for an uninfected plant at the given growth stage, regardless of inoculation status. Only later in development, above Feekes 10, did the pathogen begin overtaking the ovaries (Figure 23).

Figure 23: Morphological distinctions between emerging heads of 'Heines VII'. Upper; infected with T. caries race T-34. Lower; uninfected. Developing spikelets have been cut with a razor to reveal the interior of the spikelet, which for the infected plant is the developing sorus and the mass of developing black teliospores. For the uninfected head, only developing kernels are visible.



	Heines VII inoculated with *T. laevis* race L-18	Yayla 305 inoculated with *T. laevis* race L-18	Heines VII inoculated with *T. caries* race T-34	Yayla 305 inoculated with *T. caries* race T-34
Stage 1: Pre-Emergence	65%	79%	100%	76%
Stage 2: Emeregence	42%	53%	48%	100%
Stage 3: Two leaves	62%	80%	62%	71%
Stage 4: Second node	100%	100%	75%	33%
Expected	**(+)**	**(+)**	**(+)**	**(-)**

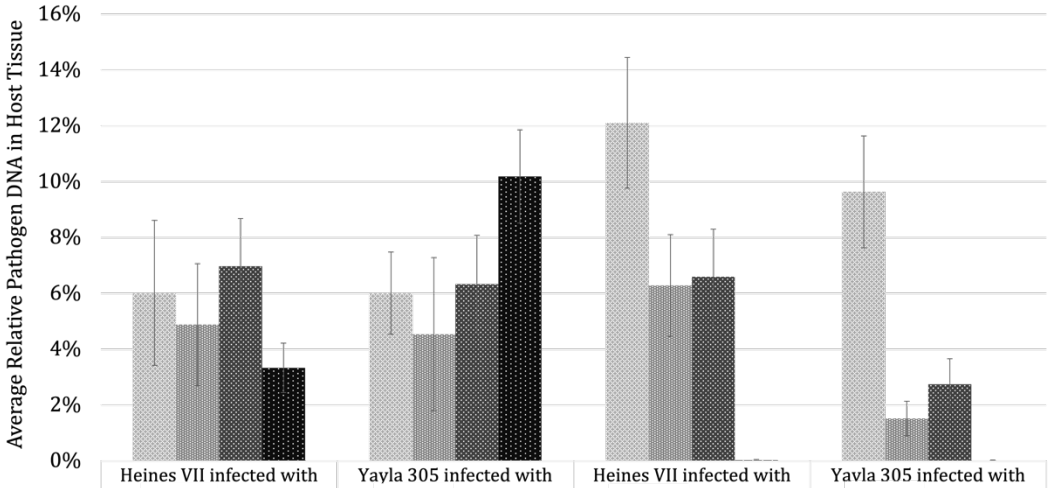
Figure 24: Colonization of plants through the developing plant as expressed by the percentage of plants that tested positive for pathogen DNA in qPCR out of the number of plants sampled in each category, contrasted with the expected results.



	Heines VII inoculated with *T. laevis* race L-18	Yayla 305 inoculated with *T. laevis* race L-18	Heines VII inoculated with *T. caries* race T-34	Yayla 305 inoculated with *T. caries* race T-34
Expected	**(+)**	**(+)**	**(+)**	**(-)**

Figure 25: Percentage of plants at maturity that had some level of sori production out of all plants rated in each category, contrasted with the expected results.

The qPCR assay was used to determine the relative pathogen DNA content in individual tissue samples. These relative values were then averaged at each growth stage for each treatment group to determine the pathogen's degree of colonization through the plant life cycle (Figure 26).



	Heines VII infected with *T. laevis* race L-18	Yayla 305 infected with *T. laevis* race L-18	Heines VII infected with *T. caries* race T-34	Yayla 305 infected with *T. caries* race T-34
Stage 1: Pre-emergence	6%	6%	12%	10%
Stage 2: Emergence	5%	5%	6%	2%
Stage 3: Two leaves	7%	6%	7%	3%
Stage 4: Second node	3%	10%	0%	0%
Expected	**(+)**	**(+)**	**(+)**	**(-)**

Figure 26: Colonization of plants through the plant lifespan as expressed by average relative pathogen DNA in host samples, contrasted with the expected results. Error bars represent standard error.

The two compatible reactions with *T. laevis* race L-18 showed fairly consistent levels of infection through the plant lifespan. They differed in that cv. 'Heines VII', with no known resistance, had less average fungal DNA relative to host DNA by the second node stage than it did at pre-emergence and emergence while *Bt8* differential cv. 'Yayla 305' showed markedly more infection at the later stage over the earlier. The incompatible reaction was not statistically different from the compatible reactions in fungal DNA concentration, which dropped dramatically by the two-leaf stage and was undetectable by the second node stage.

The model was fit based on these averages, with trial replication as a blocking factor. Overall, the model has a 1.19 ratio of the generalized χ^2 / degrees of freedom,

which is close to 1, supporting the goodness of fit of the model. The parameter estimates and p-values demonstrated that neither cultivar nor fungal race on their own explained a significant amount of variation, nor did the interaction between cultivar and fungal race. Sampling growth stage (1-4) was more significant, with a p-value of 0.0335 for the parameter itself. However, there was a strong effect of the interaction between fungal race and sampling growth stage with a p-value of 0.0350, making it hard to interpret the impacts of growth stage alone. The interaction between fungal race, cultivar, and sampling growth stage was statistically insignificant.

To interpret this model, then, it is important to identify the growth stage and fungal race for effect. Only at the fourth sampling growth stage, the differing treatment effects of race L-18 and T-34 become statistically significant. The p-value of their difference is 0.0042, indicating that the incompatible reaction and the supposedly compatible cv. 'Heines VII'/T-34 reaction are behaving differently in their progression at this stage. In looking at the averages, L-18 has an average relative pathogen DNA content of 6.76% and T-34 only 0.02%. At the second node stage, *T. caries* race T-34 has, as H.M. Woolman phrased it, "cease[d] to be a menace" (Woolman 1930), regardless of whether there is a major resistance gene present or not.

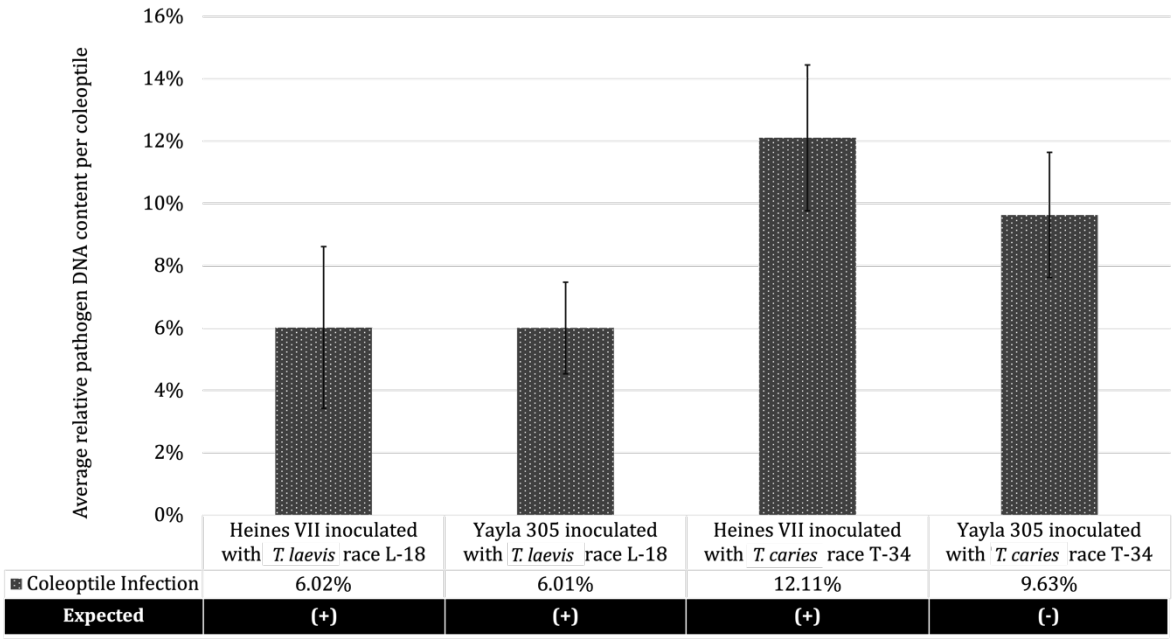


Figure 27: Average relative fungal DNA content of pre-emergent coleoptiles in separate treatment groups at the first growth stage (10-15 days post-inoculation). Error bars represent standard error.

At the first sampling growth stage, at which point the coleoptile has not yet emerged from the soil line, there is no statistical difference between the effects of either fungal race. The four treatment groups are all likewise impacted by spore germination events and colonization, despite the higher means observed for the T-34 inoculations over the L-18 inoculations.

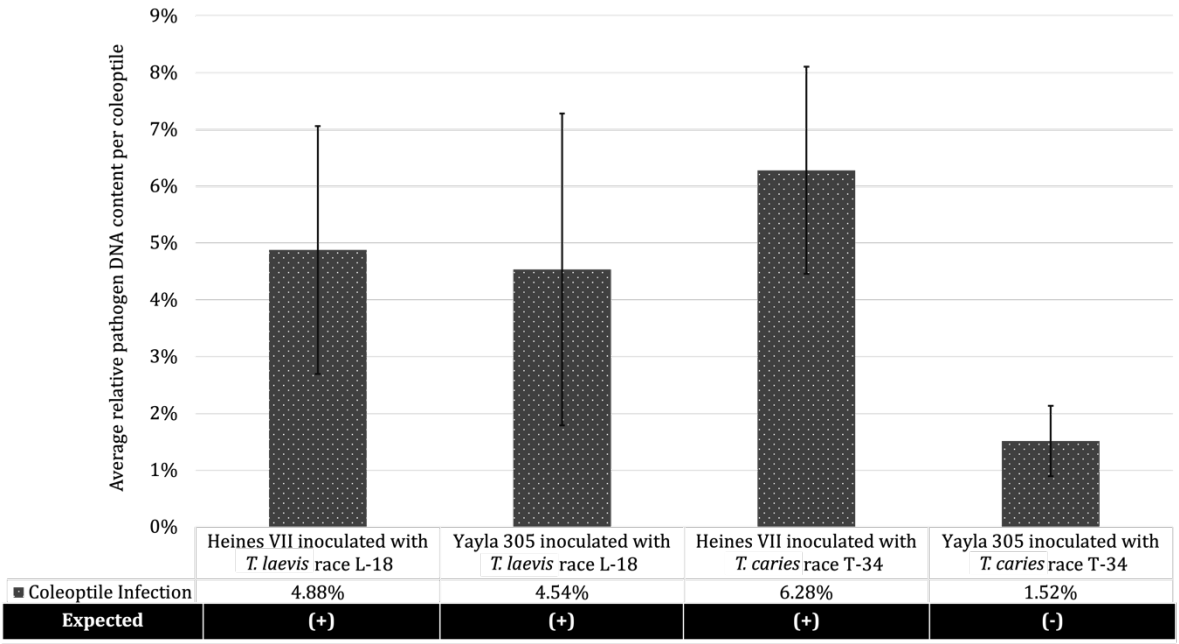
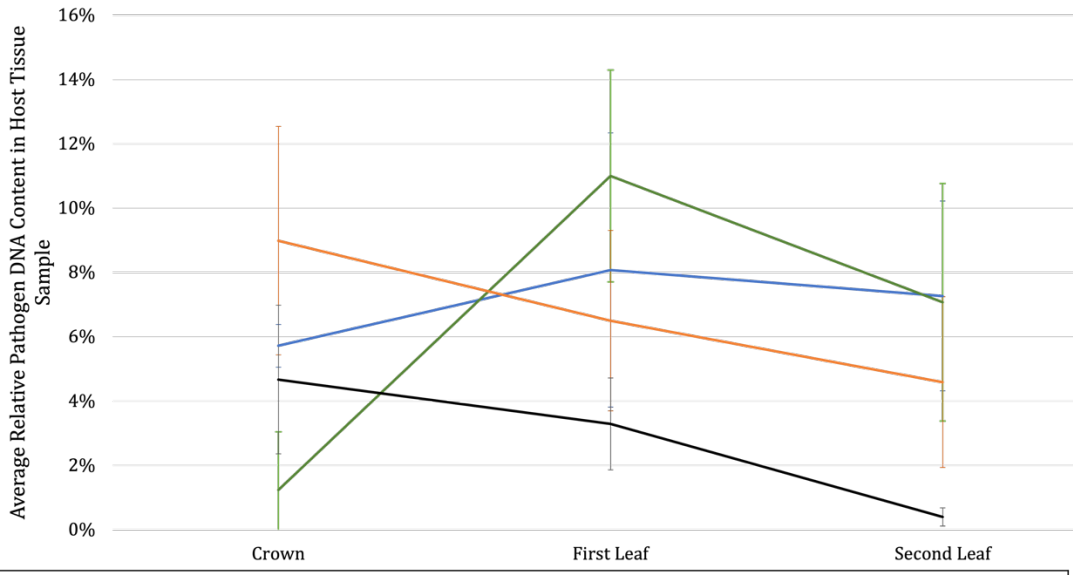


Figure 28: Average relative fungal DNA content of emerging coleoptiles in separate treatment groups at the second growth stage (15-21 days post-inoculation). Error bars represent standard error.

By the time the coleoptiles are emerging, there seems to be less pathogen DNA in samples than in the previous and later sampling growth stages in all treatment groups (Figure 28). However, within the growth stage, both races of common bunt impact both cultivars in a statistically indistinguishable manner. The difference between the lowest mean and the highest mean relative amount of fungal DNA is only 4.76%, which was insignificant as well.

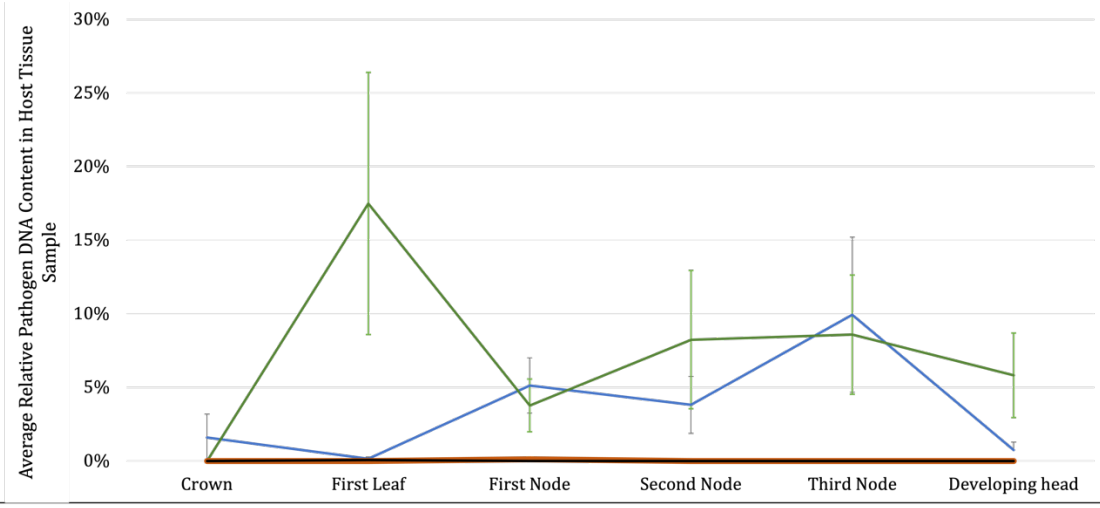


Expected Compatible	**>0%**	**>0%**	**>0%**
Heines VII inoculated with *T. laevis* race L-18	5.72%	8.08%	7.27%
Yayla 305 inoculated with *T. laevis* race L-18	1.24%	11.00%	7.08%
Heines VII inoculated with *T. caries* race T-34	8.99%	6.50%	4.59%
Expected Incompatible	**0%**	**0%**	**0%**
Yayla 305 inoculated with *T. caries* race T-34	4.67%	3.29%	0.40%

Figure 29: Progression of the pathogen through host tissues in descending order of developmental age, at the two-leaf stage (growth stage 3, 21-46 days post-inoculation) in separate treatment groups, as represented by relative pathogen DNA content in individual tissue samples. Error bars represent standard error.

By the third sampling growth stage, the two-leaf stage, plant tissues have begun differentiating. Although the tissue samples are not statistically analyzable individually due to the strong interactions of sampling growth stage and fungal race, the data itself suggests distinct colonization patterns between L-18 and T-34. It appears that L-18 maintains a stronger presence in the growing point at the crown while spreading through the first leaf and deeper into the second leaf – more fully colonizing the plant well past the infection court. T-34, however, seems to be less aggressive. In a compatible reaction between T-34 and cv. 'Heines VII' (*Bt0*), the pathogen maintains relatively high loads of pathogen DNA at the growing point, but it seems slower to make

its way to the first leaf and slower still to the second leaf. It is interesting, though, that in both the compatible and incompatible T-34 reaction the common bunt fungus does reach the inner portion of the plant, however slightly, rather than being restricted to exterior infection sites in the incompatible reaction.



Expected Compatible	**>0%**	**>0%**	**>0%**	**>0%**	**>0%**	**>0%**
Heines VII inoculated with *T. laevis* race L-18	1.59%	0.14%	5.12%	3.81%	9.94%	0.74%
Yayla 305 inoculated with *T. laevis* race L-18	0.00%	17.49%	3.77%	8.24%	8.58%	5.82%
Heines VII inoculated with *T. caries* race T-34	0.00%	0.00%	0.11%	0.00%	0.00%	0.00%
Expected Incompatible	**0%**	**0%**	**0%**	**0%**	**0%**	**0%**
Yayla 305 inoculated with *T. caries* race T-34	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%

Figure 30: Progression of the pathogen through host tissues, in descending order of developmental age, at the second node stage (growth stage 4, 119-151 days post-inoculation) in separate treatment groups, as represented by relative pathogen DNA content in individual tissue samples. Error bars represent standard error.

At the final sampling growth stage, the second node stage of the host plant's lifespan, there seems to be less of a pattern for L-18. The tissue-by-tissue progression at this growth stage was not amenable to statistical analysis due, additionally, to the large number of uninfected tissues. It seems that by this stage, the incompatible reaction has almost fully excluded T-34 from cv. 'Yayla 305' (*Bt8*) aside from the slightest trace of pathogen DNA in the first leaf. Interestingly, the reduction in fungal DNA at the second

node also occurred in the universally susceptible cultivar which averaged only 0.11% relative pathogen DNA in the first node.

Although both cultivars are well colonized by L-18, L-18 seems to progress differently between the two genotypes, more heavily and more consistently colonizing cv. 'Yayla 305'. It appears that, at this second node stage, the pathogen has grown beyond the crown and no longer maintains mycelia in that tissue. The mycelium has grown into the first leaf and the stem, where colonization has largely followed the growing point while maintaining a presence at earlier developed nodes.

L-18 may grow slower in the cv. 'Heines VII' (*Bt0*) plants, again moving out of the initially developed tissue and proceeding to colonize the rest of the plant vertically as it grows. The lack of pathogen DNA in the developing heads suggests that the pathogen has not yet colonized the developing head at the second node stage.

An additional model was constructed using PROC GLIMMIX to identify any differences between the averages of the compatible reactions and the incompatible reaction. This was undertaken to understand whether there were significant differences between reactions based only on whether or not they were known to result in disease. Overall, the model was not able to explain differences between compatible and incompatible reactions on the basis of disease reaction alone. A model was built to fit the data but was not statistically significant in total or for any parameter.

Predictive value of sampling

One of the goals of this work was to develop a correlation across genotype interactions that could be used to identify wheat genotypes that are or are not susceptible to common or dwarf bunt earlier in the life cycle than heading. To that end, plants were inoculated, and a portion of the inoculated plants were grown to maturity. Initial tests indicated that the average success of inoculations was 55% *(data not shown)*, so at least 55% diseased kernels were expected for the compatible reactions at maturity. The

incompatible reaction, though, was expected to result in no disease at maturity due to the plant's defense response.

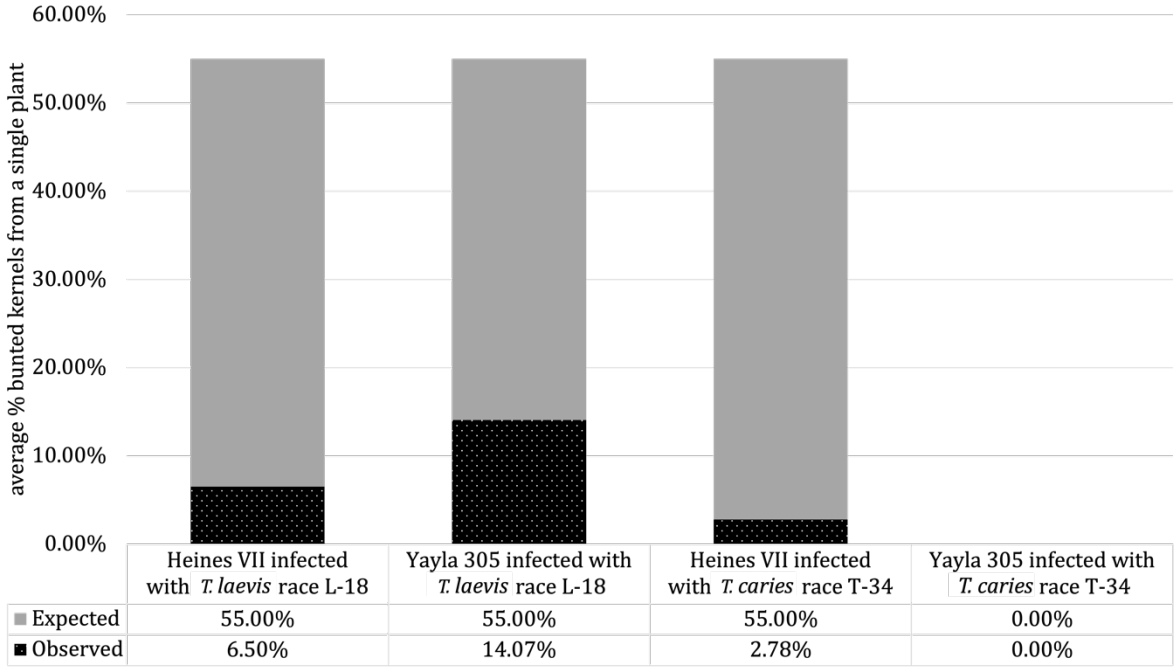


Figure 31: Bar chart of the expected vs. observed average disease outcomes of each reaction between a cultivar and specific race of common bunt

In all compatible reactions, the inoculations were much less successful at maturity than expected (Figure 31). Surprisingly, although T-34 had been characterized as the most broad-spectrum pathogenic race in aggressiveness, only an average of 2.78% of kernels on any given 'Heines VII' plant inoculated with T-34 developed sori.

The low amount of infection success led the authors to believe there might be an issue with the age of the T-34 collection, at this point 10 years old. Although herbarium studies had shown that preserved sori maintain viability for over 20 years (Fischer 1936), longevity in the field is limited to roughly two years. It was possible that the spores had lost viability, so another viability study was performed by surface sterilizing, suspending, and germinating spores from the collection. 100% of spores germinated and produced secondary sporidia and H-bodies, indicating that there was no issue with the biological material used.

Despite the low disease outcomes, the correlation between the relative amount of fungal DNA in individual samples and disease incidence was calculated to test for any useful relationships. Ideally, sampling would be non-destructive. Given that the developing head is what bears the fruit of the fungus, a relationship between the average relative fungal DNA within a treatment group within a trial and the corresponding trial-specific disease outcome was tested.

'Heines VII' inoculated with race L-18 had no fungal DNA recorded in the developing head in the 2nd node stage (*Figure 5*), but developed sori (Figure 31). 'Heines VII' inoculated with race T-34 seemed totally devoid of the pathogen at the 2nd node stage (*Figure 5*) but developed sori (Figure 31). Given these, it was unclear whether a correlation of pathogen DNA concentration in the any of the tissues to disease in developing heads could be established. It was also possible that due to the relatively high amount of pathogen DNA in the second node of cv. 'Yayla 305', the plants' first leaves infected with L-18 might be indicative of a correlation between the non-destructive sample and eventual disease outcome, given that at maturity 'Yayla 305' inoculated with race L-18 showed the greatest disease incidence.

The results of the Spearman rank correlations are found in Table 4.

Table 4: Spearman rank correlations between tissue samples of a particular treatment group in a specific replicated trial and that group's eventual disease outcome as recorded in average percent sori out of all kernels.

Growth Stage	Tissue	Spearman Correlation to Percent Bunted Kernels at Maturity
Two leaves	First leaf	0.61
Second node	First leaf	0.66
Second node	Developing head	0.67

Discussion

In this study, we aimed to clarify the procedure of common bunt colonization in both compatible and incompatible reactions through the host plant's lifespan and to use that knowledge to design an early disease screening method. Given the variability in colonization between combinations of host and pathogen genotypes, and the lack of consistency between the extent of infection in leaf samples with eventual disease incidence at maturity, early detection of resistance using qPCR seems dubious at this time. Although this study was unable to establish a correlation between measurements of relative pathogen DNA in a host tissue sample and eventual disease incidence, the results obtained provide some insight into the course of common bunt colonization.

In this study, it seems that the process of common bunt infection is not uniform between compatible reactions involving different host and pathogen genotypes. While compatible reactions involving *T. laevis* race L-18 in 'Heines VII' and 'Yayla 305' behaved similarly to each other in pre-emergent, emerging, and two-leaved plants, by the second node stage the pathogen seemed to be reacting with the different host genotypes in different ways. L-18 seemed to be able to behave more aggressively and colonize more tissues more heavily in 'Yayla 305', which has the resistance gene *Bt8*, than in the fully susceptible 'Heines VII'. 'Heines VII' also performed differently in its reactions to *T. laevis* L-18 and *T. caries* T-34. Although the genotype of the host was stable, variability in the pathogen genotype seems to create slightly different colonization patterns. 'Heines VII' inoculated with T-34 behaved more similarly to the incompatible reaction of T-34 with 'Yayla 305' than it did to Heines VII inoculated with L-18. Despite all four treatment groups responding similarly at the pre-emergence and emergence stages, by the two-leaf stage T-34 DNA had higher concentrations in the crown and first leaf of the plant than L-18, and less so the second leaf. At the second node stage, regardless of host genotype, T-34 DNA was not detected.

These results partially agree with earlier studies. The early course of infection, and its similarity between compatible and incompatible reactions, has been established and confirmed over since the 1930s (Gaudet et al. 2007; Hansen 1958; Woolman 1930).

Earlier work described a pathogen that, after germinating, searched for a weak point between two epidermal cells on the coleoptile before the coleoptile emerged from the soil, then developed an appressorium to break through the epidermis and begin the colonization process (Murray et al. 2009; Gaudet et al. 2007; Goates 1996; Woolman 1930; Sartoris 1924; Dastur 1921). Common bunt fungi were then demonstrated to colonize the first leaf base, then the second, and move through sequential leaf bases or down the leaf base to establish itself directly below the apical meristem. By the second leaf stage, some susceptible cultivars had hyphae in their first leaf bases and others had none (Swinburne 1963). The fungi then continue to infect leaf bases until establishing themselves directly beneath the growing point, and as stem elongation occurs the hyphae grow up with the growing point while the older hyphae to dissolve (Swinburne 1963; Hansen 1958). When the ear emerges, hyphae are restricted to the carpels as they begin sporulating (Swinburne 1963).

The results of this study, however, corroborate more recent studies that have disputed that understanding of colonization. Three studies were published in 2021 on the histopathology of the three closely related *Tilletia* species; one using three modes of advanced microscopy to characterize the spread of common bunt in susceptible wheat cultivars (Ren et al. 2021b), one specifically looking at the histological differences between resistant and susceptible cultivars when challenged with dwarf bunt (Xu et al. 2021), and one using qRT-PCR and laser confocal microscopy to study the progression of dwarf bunt in both resistant and susceptible cultivars (Chen et al. 2021). Rather than a small amount of colonization in older tissues, Ren et al. (2021) found the mesophyll cells ruptured at the third leaf stage, the same stage at which Xu et al. (2021) found similar deformities in the mesophyll and additionally meristematic stem cells and root cells. As the plant grew and infection spread, the pathogen was found in multiple studies throughout the roots, stems, leaf sheaths, leaf blades, glumes, and ovaries and sporulation occurred in roots, stems, leaves, glumes, and awns (Ren et al. 2021b). The structure of nuclei and chloroplasts were deleteriously altered by the infection (Ren et al. 2021b). In this study, common bunt DNA was detected in all tissues studied in compatible reactions including crowns, leaves, all nodes, and developing heads. Preliminary data [*unpublished*] collected by the authors also detected common bunt

DNA in root samples of 'Heines VII' inoculated with *T. caries*. Furthermore, this study was unlike the other recent studies in that specific *Bt* genes and common bunt races were investigated. This specification demonstrated different patterns of colonization between susceptible reactions involving different host-pathogen genetics. Our results concur with others such as Ren et al. (2021b) in painting a clear picture of an aggressive pathogen with the ability to not only colonize multiple tissues, but to alter plant structures, sporulate within different tissues, and to do so in a manner potentially specialized to the race and cultivar genetics.

In terms of incompatible reactions, at the pre-emergence growth stage there is some expectation of fungal DNA inclusion in coleoptile samples in both compatible and incompatible reactions, due to the ongoing germination events of the spores from the inoculum (Gaudet et al. 2007; Swinburne 1963; Churchward 1940; Woolman 1930). Gaudet et al. (2007) studied incompatible reactions only until the 21st day, at which point they continued to observe new spore germination events and thus new infection events. They concluded, though, that resistance is rather swift in responding to the infection event and did not observe the pathogen overcoming resistance responses at 21 days. In that case, a much lower relative pathogen DNA value would be expected in the incompatible reaction of cv. 'Yayla 305' (*Bt8*) and T-34 given that hyphae ostensibly would not be growing into the deeper parts of the coleoptile. In contrast to that expectation, there was a larger amount of relative pathogen DNA in the T-34 inoculated 'Yayla 305' (*Bt8*) plants at the pre-emergent stage than either of the L-18 compatible reactions and a comparable amount of relative pathogen DNA at the emergent stage ending at 21 days post-inoculation.

Woolman (1930) noted that while it is possible in a select few plants studied that a more, but not completely, resistant plant might harbor common bunt to 60 days, incompatible reactions usually halt the fungus by the 21st day. The two-leaf stage, occurring as late as 46 days post-inoculation, demonstrated not only a higher average amount of relative fungal DNA than would be expected were that the true standard, but also in the interior leaf, beyond the reach of new infection events. However, between the 46th day and the 119th day the pathogen is excluded in the incompatible reaction. This is similar to the findings of Xu et al. (2021) in their study of dwarf bunt infection.

They studied resistant and susceptible varieties at Zadoks 13 (three leaf stage, one stage beyond the two-leaf stage) and Zadoks 21 (main stem with one additional tiller, ten Zadoks growth stages prior to the second node), and found that between the two stages the pathogen was excluded. They did not report the days post-inoculation for each sampling time.

Without the knowledge of when exactly resistance engages in the incompatible reactions, it is more difficult to determine when a non-destructive sample would be most useful. In this study, three correlations were examined: the first leaf from the two-leaf stage, the first leaf from the second node stage, and the developing head from the second node stage (Table 4). None of the correlations at this stage were particularly strong, all were about 0.6. This indicates that there may be a positive correlation, but the analysis is hindered by the low disease pressure. Without high disease pressure, the points where a higher relative pathogen DNA amount and a higher average percent disease incidence are outliers and would skew a linear regression. Therefore, a Spearman rank correlation must be used. At this time, no diagnostic assay could be developed from the data, but ruling out the possibility requires further study.

While this study did not examine a segregating population for evidence of quantitative disease resistance, previous research has suggested that phenotypic distributions of susceptibility may not be explicable by major resistance genes (Pope and Dewey 1975). Quantitative disease resistance confers a reduction in disease rather than an exclusion of disease (St. Clair 2010). Our results showed an exclusion of disease in the incompatible reaction, though that was preceded by proliferation of the fungus in earlier growth stages. Coupled with a low level of infection at maturity across all treatment groups, it is surprising that T-34 was able to persist in *Bt8* differential 'Yayla 305' past the infection court, beyond the spatial influence of new infection events. This may suggest some degree of quantitative resistance associated with *Bt8*. *Bt10*, as studied by Gaudet et al. (2007) seems to work with both *T. caries* T-1 and T-27 in the same way. It creates a bipartite reaction zone, where the entering fungus is cased in callose. The results were fairly immediate. However, there has been some speculation that *Bt8* may be a heritable linkage of genes, which might act more quantitatively. If quantitative disease resistance can be observed in a reduction of disease rather than

the presence or absence of disease, then the frequent occurrence of incompatible reactions producing small amounts of disease (<10%), and the variability in the infection outcomes of compatible reactions, suggests that there may be other quantitative trait loci at play. It is possible that if disease pressure in this study had been higher, we may have seen more of a range of disease outcomes such as those that have been observed in the past. Additionally, race-specificity of resistance can be an outcome of quantitative disease resistance (St. Clair 2010). Our observations suggest that resistance and susceptibility between common bunt fungi and wheat is contextualized by and dependent on both the host and pathogen genotype, which could be a feature of quantitative disease resistance. To add strength to these findings, multiple QTL have been identified in different wheat cultivars that address common and dwarf bunt resistance (Muellner et al. 2020b, 2020a; Wang et al. 2019; Bokore et al. 2019; Chen et al. 2016; Wang et al. 2009; Fofana et al. 2008). It is highly likely that, given the discovery of many QTL, quantitative resistance influenced the phenotypic reactions in this study. The low disease pressure, though, prohibits clear discussion.

Gaudet et al.'s (2007) study of *Bt10* concluded both that the common bunt pathogen was not subject to a systemic response and, if it were, would be unable to overcome it. They explained low levels of infection in incompatible reactions as an environmentally-mediated breakdown of resistance. While it is known that *Bt8* resistance can be broken down in certain environmental conditions (Gaudet and Menzies 2012), it is unusual for a susceptible variety to become more resistant. In this study, the universal suscept Heines VII was less diseased than the *Bt8* differential Yayla 305 when challenged with L-18, and much less susceptible to the broad-spectrum virulent race T-34 than it was to L-18. It is likely that there are environmental factors that affect the "resistant" phenotype, and although the author followed carefully the methods on germinating and vernalizing the plants, found a lack of literature on the exact method of increasing temperatures in the growth chamber after vernalization. Many of the pants experienced heat stress at that time, which is expected to have reduced the viability of the pathogen

As is the case with this potential environmentally-mediated resistance, some other findings were unexpected as well. In the statistical model, the interaction of

pathogen race and sampling time was statistically significant, but the three-way interaction of these two parameters and the cultivar was not. This could be because cultivar responses in the early growth stages are very similar. Additionally, the impacts of compatibility vs. incompatibility were totally insignificant when modeled. This is likely due to the lack of similarity in the progression of "compatible reactions," precluding a single progression of a "compatible" reaction or an "incompatible" reaction.

As opposed to the conclusions of preceding research, and in concert with more recent work, this study found that in the expected incompatible interaction of *T. caries* race and T-34, the fungus colonized the *Bt8* differential up to 46 days, and at some point before 119 days the plant completely excluded the pathogen. This was much different than what was observed for interactions between T-1 and T-27 with *Bt10.* This shifts our understanding of wheat resistance and tolerance to common bunt, suggesting that separate major resistance genes act differently from each other to exclude the pathogen and may be joined by minor resistance genes to do so. The compatible reactions showed no unanimous pattern, suggesting that not all compatible reactions progress in the same way. Overall, it seems that major resistance genes are an unlikely explanation of all the observations. Further study of more resistance genotypes may yet reveal alternate modes of resistance, and likely many minor resistance genes are yet to be described.

If different genes do, in fact, have different modes-of-action, then it becomes possible to utilize different genetics to pyramid genes more intentionally to create more durable resistance. It also necessitates more information to be reported from future studies. This information makes it no longer sufficient to report "common bunt" or "dwarf bunt" in histopathological studies, but instead requires the disclosure of the race(s) used, or, at the very least, the origin of the isolates. Authors must also report the resistance genotypes of the host plants they work with, and the manner and timing of sampling.

Given that the exact timing of the resistance action was not discerned in this study, marker-assisted selection (MAS) may still be a more appropriate tool for breeders than earlier non-destructive qPCR diagnostics. Therefore, future study should

continue to elucidate markers for common and dwarf bunt resistance. MAS is less useful until many additional markers have been identified, so in the meantime more work should be done to develop the assay used in this study regarding the timing of non-destructive sampling.

Future work will also need to approach the study of resistance from the pathogen perspective. There is scant literature on any *Avr* genes that may be possessed by the common bunt fungi themselves, which is a glaring hole in our understanding of the pathosystem. Recent work has tested the interactome of wheat and common bunt *in silico*, identifying 648 likely effectors in their *T. caries* genome and 575 in *T. laevis* (Kataria and Kaundal 2022). These remain to be studied in detail, though. Ongoing work on common bunt is necessary as it continues to grow in its threat, and research has yet to be done to fully understand the complexities of this pathogen. The research presented here attempts to answer questions of the pathogen colonization but opens many more questions about the specificities of the interactions.

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Chapter 4:
A summary of the work on common bunt of winter wheat

Common bunt was, historically, one of the most devastating diseases of wheat in the world. With the disease endemic to every wheat-growing region, and without seed protectants available, management relied fully on resistance. While resistant varieties were very successful, the nature of fungi is to mutate and/or hybridize which may result in the fungi overcoming resistance genes more quickly than resistance may be bred into new varieties. In the 1950s, seed treatment fungicides were introduced to address the disease. Many additional fungicides have been released, affording excellent control in treated fields, yet the problem persists where treatment is inaccessible or impermissible such as in organic production.

To address the ongoing threat of common bunt, breeding for resistance continues. Resistance breeding relies on rating for disease using classic methods – counting the number of diseased heads in a stand. This requires specialized knowledge of the pathosystem and can be quite subjective and variable between observers. In recent years, some study has focused on the discovery and description of quantitative trait loci (QTL) for marker-assisted selection (MAS) (Muellner et al. 2020b, 2020a; Bokore et al. 2019; Steffan et al. 2017; Zou et al. 2017; Singh et al. 2016; Dumalasová et al. 2012; Fofana et al. 2008). Effective development of MAS can remove the need to inoculate and grow all candidate germplasm out to heading. However, it may take years to build up the appropriate knowledge of markers for all the known major resistance genes and significant minor QTL.

Our understanding of the mechanisms of resistance in wheat to common bunt is rather limited. There are 16 major resistance genes that have been identified, but only one had been specifically studied for its action *in planta*. The conclusions of that study were that resistance is swift and non-systemic, and any faltering in major resistance was likely due to environmentally induced breakdown of resistance (Gaudet et al. 2007). Previous research on incompatible reactions, in which resistance was studied

but without a focus on any specific known resistance gene, made similar conclusions (Hansen 1958; Woolman 1930). Although Woolman (1930) found fungi in some moderately resistant plants up to 60 days post-inoculation, he concluded that resistance was generally swift as, of the three successive phases infection could be divided into, only the first was ever seen in incompatible reactions. Gaudet et al. (2007) reported that Swinburne's (1930) cultivars likely harbored resistance genes *Bt1* and *Bt7*, although at the time the genotype had not been discerned. Gaudet et al. (2007) used a *Bt10* differential in studying host resistance. Common bunt consists of a classification ranking to the race level, in which different races are characterized by their reactions to the set of 16 single-resistance gene differentials. Resistance is highly race-specific, and different geographic locations host different combinations of common bunt races. Gaudet et al. (2007) studied *Bt10* due to the effective use in Canada, where their research was performed. In the Pacific Northwest, *Bt8* has been the most durable and successful resistance gene (Goates 2012). The question then remained, given that resistance is highly race-specific, would *Bt8* act differently than *Bt10* and *Bt1*/*Bt7*, and if so would combining *Bt8* with other known resistance genes maintain or increase the durability of resistance? This study tried to address that question, and to develop a new qPCR method that might also prove useful in reducing variability in disease rating and shorten the timeline for breeding new resistant varieties by providing an earlier confirmation of infection in asymptomatic tissue.

In Chapter 2, the qPCR assay that was used was previously published. Using existing primers and probes for the common/dwarf bunt internal transcribed spacer (ITS) region (Zgraja et al. 2016), existing primers for the wheat *β-actin* gene (Wei et al. 2015), and a newly developed probe for the *β-actin* gene the assay was developed within the specifications of the Universal Express qPCR SuperMix (Invitrogen, Waltham, MA) to create a rapid, high-throughput protocol sensitive to 0.1 pg of pathogen DNA. The assay does not require a standard curve, provides a relative quantitative measure of the extent of pathogen colonization in a tissue, and is designed for use with directly sampled plant tissue instead of spores or cultures.

In Chapter 3, the qPCR assay is used to explore differences in the progression of common bunt colonization between two cultivars of wheat with different resistance

genetics: *Triticum aestivum* cv. 'Heines VII' (lacking known resistance genes) and PI178210 cv. 'Yayla 305' (possessing the *Bt8* resistance gene). Two races of common bunt were used, one a race of *T. laevis* and the other of *T. caries*. *T. laevis* race L-18 was used to study the compatible reactions in both 'Heines VII' and 'Yayla 305.' *T. caries* race T-34 is virulent to 'Heines VII' and avirulent to *Bt8*, and so was used to study the differences in the compatible and incompatible reaction. Samples of individually excised tissues were taken at four growth stages: (1) pre-emergence, (2) emergence, (3) two-leaves, and (4) second node. The results of this study were that, as previously documented, compatible and incompatible reactions result in the same initial colonization of the seedling, regardless of host or pathogen genotype which continues into the two-leaf stage. At the two-leaf stage, previous studies suggested that the host would exclude the common bunt pathogens in incompatible reactions. Pathogen DNA was detected in not only the crown and first leaves but the second leaf as well. The second leaf would be beyond the access of new infection events from spores germinating in the soil, suggesting that *Bt8* resistance is not as swift as *Bt10*. By the second node stage, significant differences appear between reactions that seem guided by both host and pathogen genetics. In general, in compatible reactions plant tissue seems to be more thoroughly colonized by fungal hyphae than previous 20th century studies had suggested, which agreed with several 2021 studies on common and dwarf bunt (Chen et al. 2021; Ren et al. 2021b; Xu et al. 2021).

Non-destructively sampled leaves were taken from plants at the two-leaf and second-node stages to look for a correlation between fungal growth early in the infection cycle and eventual disease outcome. Unfortunately, Spearman rank correlations for the relationships were roughly 0.6 which does not allow for a confident reporting of a correlation but does suggest that there is some relationship worth further exploration. The disease pressure in this experiment was low, but under higher disease pressure a repeat of this experiment might yield a higher correlation as a wider range of outcomes might be observed. Different non-destructive tissues, such as the flag leaf, might be more meaningfully infected in terms of predictive value and would develop past the point at which the resistant reaction begins. Later development might avoid complications introduced by the similarity in compatible and incompatible

reactions up to 46 days post-inoculation, while still removing weeks from the breeding timeline.

Through the course of the project, challenges were encountered and occasionally results were surprising. While standard methodology was used for inoculations, the author failed to infer the necessity of surface sterilization of seeds. As a consequence, Fusarium crown rot unknowingly present on the seeds developed into disease fairly uniformly beginning at the first node stage. Mortality was limited, but disease was widespread. The symptoms of common bunt and Fusarium crown rot are easily distinguishable, and the qPCR assay used has been tested against all causal *Fusarium* species and showed no cross-reactions (Zgraja et al. 2016). While it is possible that co-infection may have modified common bunt pathogenesis, it would have done so uniformly, and it is highly unlikely that the two pathogens were confused for each other. In future work, it is recommended that all seeds be surface sterilized and dried prior to inoculation with common bunt.

Additionally, common bunt disease pressure in these experiments was unexpectedly low. A protocol modified from Goates (2012) was used, and any of these modifications may have reduced the effectivity of inoculations. A lower concentration of methyl cellulose was prepared from powdered methyl cellulose, rather than a higher concentration prepared from a liquid stock. It is possible that a higher viscosity methyl cellulose solution may have increased the adherence of the spores to the seed coats, leaving them in closer proximity to infect once germinated. The soil media was unspecified in the Goates (2012) paper, though previous literature has established that common bunt fungi germinate best in a neutral "mineral soil with a clay base" and high humus (Fischer and Holton 1957). The soil mix used in this study was a sandy soil that, though it had a high humus content due to the large proportion of peat moss, likely was fairly acidic and was watered with a hard water source. This may have been less than ideal for germination of spores. While spore adhesion and soil texture may have introduced some challenges for infection success, qPCR measurements at pre-emergence and emergence showed a high proportion of plants infected. It is most likely that the author's interpretation of "gradually warm[ing] to approximately 30°C during the day and 18°C during the night" (Goates 2012) was overly rapid, and the heat

shocking of the plants negatively impacted the ability of the infecting common bunt fungi to cause disease. In future studies, it would be recommended to more closely follow established protocols and to recreate as best as possible the environmental conditions conducive to common bunt. This would include mimicking the ideal soil texture, using more neutral and less ionized water, and mimicking greenhouse conditions in growth chambers more closely by using a gradient protocol to increase and decrease light and warming up the chamber less rapidly initially.

The conclusions of this study, that infection progresses differently in the presence of different resistance genes and pathogenic races, are promising for future work. The qPCR assay herein described will be useful in further study of histopathology, as it provides good initial results for locating the fungus without the need for time-consuming microscopic exploration. The addition of microscopic observation in tissues that test positive for common bunt will create a clear picture of infection that, due to the specific nature of the qPCR primers, will confirm hyphae as common bunt and not a contaminant or other endophytic fungal species. To improve and validate the qPCR assay, future work should focus on reducing the expense involved with the reagents for DNA extraction and thermal cycling and verifying the stability of positive results across multiple pathogen races and multiple host genotypes.

While all previous research points to co-relevance between research on common and dwarf bunt, this work suggests that there may be significant differences in the way different races of common bunt infect the same wheat host. Given that the primers used in the qPCR assay are specific to both common and dwarf bunt pathogens, this assay may be useful in the study of dwarf bunt as well to discern how *Tilletia controversa* proceeds with infection. The differences in the reactions of different resistance genes with different fungal races may lead to discoveries on the most useful pyramiding of resistance genes to create the most durable resistance to both common and dwarf bunt, thereby improving our ability to farm wheat organically, protect volunteer, reduce spore loads in the soil, and secure international trade of grain.

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Appendix I: SAS Code Used for Data Analysis

For the Generalized Linear Mixed Model, Using PROC GLIMMIX:

```
* input data **/;

data cov;
input sample  treatment$  cultivar$  reaction$  tissue$  plantnum trial
      gs  zadoks feekes
dpi   fdna;
cards;

315  L18  HeinesVII  Compatible  Coleoptile  1  1  1  5  0
     11  0.089006237
359  L18  HeinesVII  Compatible  Coleoptile  4  1  1  5  0
     13  0.038843709
357  L18  HeinesVII  Compatible  Coleoptile  3  1  1  7  0
     13  1.00E-08
363  L18  HeinesVII  Compatible  Coleoptile  2  1  2  10  1
     18  1.00E-08
383  L18  HeinesVII  Compatible  Coleoptile  3  1  2  10  1
     19  1.00E-08
387  L18  HeinesVII  Compatible  Coleoptile  5  1  2  10  1
     19  1.00E-08
361  L18  HeinesVII  Compatible  Coleoptile  1  1  2  11  1
     19  0.066877498
491  L18  HeinesVII  Compatible  Crown 1  1  3  12  1  36
     1.00E-08
492  L18  HeinesVII  Compatible  FirstLeaf  1  1  3  12  1
     36  1.00E-08
```

493	L18	HeinesVII	Compatible	SecondLeaf	1	1	3	12	1
36	1.00E-08								
495	L18	HeinesVII	Compatible	Crown 2	1	3	12	1	36
1.00E-08									
496	L18	HeinesVII	Compatible	FirstLeaf	2	1	3	12	1
36	0.009311363								
497	L18	HeinesVII	Compatible	SecondLeaf	2	1	3	12	1
36	0.054584429								
499	L18	HeinesVII	Compatible	Crown 3	1	3	12	1	36
1.00E-08									
500	L18	HeinesVII	Compatible	FirstLeaf	3	1	3	12	1
36	1.00E-08								
501	L18	HeinesVII	Compatible	SecondLeaf	3	1	3	12	1
36	1.00E-08								
503	L18	HeinesVII	Compatible	Crown 4	1	3	12	1	36
0.007193705									
504	L18	HeinesVII	Compatible	FirstLeaf	4	1	3	12	1
36	0.470177943								
505	L18	HeinesVII	Compatible	SecondLeaf	4	1	3	12	1
36	1.00E-08								
507	L18	HeinesVII	Compatible	Crown 5	1	3	12	1	36
0.007868506									
508	L18	HeinesVII	Compatible	FirstLeaf	5	1	3	12	1
36	1.00E-08								
509	L18	HeinesVII	Compatible	SecondLeaf	5	1	3	12	1
36	1.00E-08								
365	L18	Yayla305	Compatible	Coleoptile	1	1	1	5	0
13	0.02355769								
371	L18	Yayla305	Compatible	Coleoptile	5	1	1	5	0
15	0.001322269								

367	L18	Yayla305	Compatible	Coleoptile	2	1	1	9	0
	12	0.054761566							
369	L18	Yayla305	Compatible	Coleoptile	4	1	1	9	0
	14	0.035268999							
373	L18	Yayla305	Compatible	Coleoptile	3	1	1	9	0
	13	0.009743013							
375	L18	Yayla305	Compatible	Coleoptile	1	1	2	10	1
	19	1.00E-08							
377	L18	Yayla305	Compatible	Coleoptile	2	1	2	10	1
	19	1.00E-08							
379	L18	Yayla305	Compatible	Coleoptile	3	1	2	10	1
	19	1.00E-08							
381	L18	Yayla305	Compatible	Coleoptile	4	1	2	10	1
	19	0.064565639							
401	L18	Yayla305	Compatible	Coleoptile	5	1	2	10	1
	19	1.00E-08							
479	L18	Yayla305	Compatible	Crown 3	1	3	12	1	36
	1.00E-08								
480	L18	Yayla305	Compatible	FirstLeaf	3	1	3	12	1
	36	1.00E-08							
481	L18	Yayla305	Compatible	SecondLeaf	3	1	3	12	1
	36	1.00E-08							
485	L18	Yayla305	Compatible	SecondLeaf	4	1	3	12	1
	36	0.224863258							
487	L18	Yayla305	Compatible	Crown 5	1	3	12	1	36
	1.00E-08								
488	L18	Yayla305	Compatible	FirstLeaf	5	1	3	12	1
	36	0.093238895							
489	L18	Yayla305	Compatible	SecondLeaf	5	1	3	12	1
	36	1.00E-08							

475	L18	Yayla305	Compatible	Crown 2	1	3	13	1	36
	1.00E-08								
476	L18	Yayla305	Compatible	FirstLeaf	2	1	3	13	1
	36	0.002116581							
477	L18	Yayla305	Compatible	SecondLeaf	2	1	3	13	1
	36	1.00E-08							
472	L18	Yayla305	Compatible	FirstLeaf	1	1	3	.	1
	35	0.456049832							
473	L18	Yayla305	Compatible	SecondLeaf	1	1	3	.	1
	35	1.00E-08							
345	T34	HeinesVII	Compatible	Coleoptile	3	1	1	5	0
	15	0.096308226							
341	T34	HeinesVII	Compatible	Coleoptile	2	1	1	7	0
	14	0.03532189							
347	T34	HeinesVII	Compatible	Coleoptile	4	1	1	7	0
	15	0.288794495							
343	T34	HeinesVII	Compatible	Coleoptile	1	1	1	9	0
	14	0.000604007							
351	T34	HeinesVII	Compatible	Coleoptile	5	1	1	9	0
	15	0.099786494							
389	T34	HeinesVII	Compatible	Coleoptile	1	1	2	10	1
	19	0.098734537							
393	T34	HeinesVII	Compatible	Coleoptile	3	1	2	10	1
	19	0.08965319							
395	T34	HeinesVII	Compatible	Coleoptile	4	1	2	10	1
	19	0.111968627							
409	T34	HeinesVII	Compatible	Coleoptile	5	1	2	10	1
	19	0.258676488							
451	T34	HeinesVII	Compatible	Crown 1	1	3	12	1	36
	0.023765481								

452	T34	HeinesVII	Compatible	FirstLeaf	1	1	3	12	1
36	0.183080693								
453	T34	HeinesVII	Compatible	SecondLeaf	1	1	3	12	1
36	0.001018307								
455	T34	HeinesVII	Compatible	Crown 2	1	3	12	1	36
0.43301066									
457	T34	HeinesVII	Compatible	SecondLeaf	2	1	3	12	1
36	0.048576089								
460	T34	HeinesVII	Compatible	FirstLeaf	3	1	3	12	1
36	1.00E-08								
461	T34	HeinesVII	Compatible	SecondLeaf	3	1	3	12	1
36	0.010156836								
464	T34	HeinesVII	Compatible	FirstLeaf	4	1	3	12	1
36	1.00E-08								
465	T34	HeinesVII	Compatible	SecondLeaf	4	1	3	12	1
36	1.00E-08								
467	T34	HeinesVII	Compatible	Crown 5	1	3	12	1	36
0.241055446									
469	T34	HeinesVII	Compatible	SecondLeaf	5	1	3	12	1
36	1.00E-08								
313	T34	Yayla305	Incompatible	Coleoptile	1	1	1	5	0
11	0.01773527								
323	T34	Yayla305	Incompatible	Coleoptile	2	1	1	7	0
12	0.023619215								
333	T34	Yayla305	Incompatible	Coleoptile	5	1	1	7	0
12	0.037116968								
325	T34	Yayla305	Incompatible	Coleoptile	3	1	1	9	0
14	1.00E-08								
329	T34	Yayla305	Incompatible	Coleoptile	4	1	1	9	0
12	0.018927584								

339	T34	Yayla305	Incompatible	Coleoptile	4	1	2	10	1	19	0.000353288
327	T34	Yayla305	Incompatible	Coleoptile	1	1	2	11	1	19	0.010470037
331	T34	Yayla305	Incompatible	Coleoptile	2	1	2	11	1	19	0.081450961
335	T34	Yayla305	Incompatible	Coleoptile	3	1	2	11	1	19	0.004789482
407	T34	Yayla305	Incompatible	Coleoptile	5	1	2	11	1	19	0.002167546
431	T34	Yayla305	Incompatible	Crown 1	1	3	12	1	36	1.00E-08	
432	T34	Yayla305	Incompatible	FirstLeaf	1	1	3	12	1	36	0.064582774
433	T34	Yayla305	Incompatible	SecondLeaf	1	1	3	12	1	36	1.00E-08
435	T34	Yayla305	Incompatible	Crown 2	1	3	12	1	36	1.00E-08	
436	T34	Yayla305	Incompatible	FirstLeaf	2	1	3	12	1	36	1.00E-08
437	T34	Yayla305	Incompatible	SecondLeaf	2	1	3	12	1	36	0.008103055
447	T34	Yayla305	Incompatible	Crown 5	1	3	12	1	36	0.30887732	
448	T34	Yayla305	Incompatible	FirstLeaf	5	1	3	12	1	36	0.071829268
449	T34	Yayla305	Incompatible	SecondLeaf	5	1	3	12	1	36	1.00E-08
439	T34	Yayla305	Incompatible	Crown 3	1	3	.	1	35	0.396782073	

441	T34	Yayla305	Incompatible	SecondLeaf	3	1	3	.	1
	35	1.00E-08							
443	T34	Yayla305	Incompatible	Crown	4	1	3	.	1
35	0.012364405								
444	T34	Yayla305	Incompatible	FirstLeaf	4	1	3	.	1
	35	1.00E-08							
445	T34	Yayla305	Incompatible	SecondLeaf	4	1	3	.	1
	35	1.00E-08							
511	L18	HeinesVII	Compatible	Coleoptile	1	2	1	7	0
	13	1.00E-08							
513	L18	HeinesVII	Compatible	Coleoptile	2	2	1	7	0
	14	0.035968317							
515	L18	HeinesVII	Compatible	Coleoptile	3	2	1	7	0
	14	0.117901453							
519	L18	HeinesVII	Compatible	Coleoptile	5	2	1	7	0
	14	0.085749469							
521	L18	HeinesVII	Compatible	Coleoptile	6	2	1	7	0
	13	0.032139065							
523	L18	HeinesVII	Compatible	Coleoptile	7	2	1	7	0
	14	0.045601238							
525	L18	HeinesVII	Compatible	Coleoptile	8	2	1	7	0
	13	0.527994298							
527	L18	HeinesVII	Compatible	Coleoptile	9	2	1	7	0
	13	0.088687173							
595	L18	HeinesVII	Compatible	Coleoptile	1	2	2	11	1
	19	0.035103712							
597	L18	HeinesVII	Compatible	Coleoptile	2	2	2	11	1
	20	0.051485385							
599	L18	HeinesVII	Compatible	Coleoptile	3	2	2	11	1
	20	0.027498786							

603	L18	HeinesVII	Compatible	Coleoptile	5	2	2	11	1
	20	0.131665773							
605	L18	HeinesVII	Compatible	Coleoptile	6	2	2	11	1
	20	0.127652192							
607	L18	HeinesVII	Compatible	Coleoptile	7	2	2	11	1
	20	1.00E-08							
609	L18	HeinesVII	Compatible	Coleoptile	8	2	2	11	1
	20	0.413984491							
611	L18	HeinesVII	Compatible	Coleoptile	9	2	2	11	1
	20	0.121317521							
727	L18	HeinesVII	Compatible	Crown 4	2	3	11	1	35
	0.212821496								
729	L18	HeinesVII	Compatible	SecondLeaf	4	2	3	11	1
	35	0.020571815							
739	L18	HeinesVII	Compatible	Crown 6	2	3	11	1	35
	0.068628999								
740	L18	HeinesVII	Compatible	FirstLeaf	6	2	3	11	1
	35	0.11033498							
741	L18	HeinesVII	Compatible	SecondLeaf	6	2	3	11	1
	35	0.033067166							
747	L18	HeinesVII	Compatible	Crown 8	2	3	11	1	35
	0.065808225								
748	L18	HeinesVII	Compatible	FirstLeaf	8	2	3	11	1
	35	0.246685517							
749	L18	HeinesVII	Compatible	SecondLeaf	8	2	3	11	1
	35	0.033654215							
723	L18	HeinesVII	Compatible	Crown 3	2	3	12	1	39
	0.188398601								
725	L18	HeinesVII	Compatible	SecondLeaf	3	2	3	12	1
	39	0.600102543							

743	L18	HeinesVII	Compatible	Crown 7	2	3	12	1	35
	0.215736234								
744	L18	HeinesVII	Compatible	FirstLeaf	7	2	3	12	1
	35	0.075143629							
745	L18	HeinesVII	Compatible	SecondLeaf	7	2	3	12	1
	35	1.00E-08							
715	L18	HeinesVII	Compatible	Crown 1	2	3	13	1	35
	1.00E-08								
716	L18	HeinesVII	Compatible	FirstLeaf	1	2	3	13	1
	35	1.00E-08							
717	L18	HeinesVII	Compatible	SecondLeaf	1	2	3	13	1
	35	1.00E-08							
719	L18	HeinesVII	Compatible	Crown 2	2	3	13	1	35
	1.00E-08								
720	L18	HeinesVII	Compatible	FirstLeaf	2	2	3	13	1
	35	1.00E-08							
721	L18	HeinesVII	Compatible	SecondLeaf	2	2	3	13	1
	35	1.00E-08							
735	L18	HeinesVII	Compatible	Crown 5	2	3	13	1	35
	1.00E-08								
736	L18	HeinesVII	Compatible	FirstLeaf	5	2	3	13	1
	35	1.00E-08							
737	L18	HeinesVII	Compatible	SecondLeaf	5	2	3	13	1
	35	1.00E-08							
1202	L18	HeinesVII	Compatible	Crown 1	2	5	32	7	
	149	1.00E-08							
1203	L18	HeinesVII	Compatible	FirstNode	1	2	5	32	7
	149	0.17176919							
1205	L18	HeinesVII	Compatible	SecondNode	1	2	5	32	7
	149	0.225874753							

1206	L18	HeinesVII	Compatible	ThirdNode	1	2	5	32	7
	149	0.292113665							
1207	L18	HeinesVII	Compatible	Devhead	1	2	5	32	7
	149	0.041083586							
1208	L18	HeinesVII	Compatible	FirstNode	1	2	5	32	7
	149	0.115699891							
1211	L18	HeinesVII	Compatible	ThirdNode	1	2	5	32	7
	149	0.196363204							
1212	L18	HeinesVII	Compatible	FirstNode	1	2	5	32	7
	149	0.141761694							
1214	L18	HeinesVII	Compatible	SecondNode	1	2	5	32	7
	149	0.263361323							
1291	L18	HeinesVII	Compatible	FirstNode	2	2	5	32	7
	151	1.00E-08							
1292	L18	HeinesVII	Compatible	FirstLeaf	2	2	5	32	7
	151	1.00E-08							
1293	L18	HeinesVII	Compatible	SecondNode	2	2	5	32	7
	151	1.00E-08							
1294	L18	HeinesVII	Compatible	Devhead	2	2	5	32	7
	151	1.00E-08							
1286	L18	HeinesVII	Compatible	FirstNode	2	2	5	39	9
	151	1.00E-08							
1287	L18	HeinesVII	Compatible	FirstLeaf	2	2	5	39	9
	151	1.00E-08							
1288	L18	HeinesVII	Compatible	SecondNode	2	2	5	39	9
	151	1.00E-08							
1289	L18	HeinesVII	Compatible	ThirdNode	2	2	5	39	9
	151	1.00E-08							
1290	L18	HeinesVII	Compatible	Devhead	2	2	5	39	9
	151	1.00E-08							

549	L18	Yayla305	Compatible	Coleoptile	2	2	1	7	0
	14	0.076668532							
553	L18	Yayla305	Compatible	Coleoptile	4	2	1	7	0
	13	0.096983594							
555	L18	Yayla305	Compatible	Coleoptile	5	2	1	7	0
	14	0.046071771							
557	L18	Yayla305	Compatible	Coleoptile	6	2	1	7	0
	14	0.103232865							
559	L18	Yayla305	Compatible	Coleoptile	7	2	1	7	0
	14	0.189620557							
561	L18	Yayla305	Compatible	Coleoptile	8	2	1	7	0
	14	0.245508342							
563	L18	Yayla305	Compatible	Coleoptile	9	2	1	7	0
	14	0.080379624							
635	L18	Yayla305	Compatible	Coleoptile	3	2	2	10	1
	20	0.027845727							
631	L18	Yayla305	Compatible	Coleoptile	1	2	2	11	1
	20	0.552350996							
633	L18	Yayla305	Compatible	Coleoptile	2	2	2	11	1
	19	0.038080514							
637	L18	Yayla305	Compatible	Coleoptile	4	2	2	11	1
	20	0.072093875							
639	L18	Yayla305	Compatible	Coleoptile	5	2	2	11	1
	20	1.00E-08							
641	L18	Yayla305	Compatible	Coleoptile	6	2	2	11	1
	20	0.087397457							
645	L18	Yayla305	Compatible	Coleoptile	8	2	2	11	1
	20	0.053860038							
647	L18	Yayla305	Compatible	Coleoptile	9	2	2	11	1
	20	0.010840555							

679	L18	Yayla305	Compatible	Crown 1	2	3	12	1	35
	1.00E-08								
680	L18	Yayla305	Compatible	FirstLeaf	1	2	3	12	1
	35	1.00E-08							
681	L18	Yayla305	Compatible	SecondLeaf	1	2	3	12	1
	35	1.00E-08							
689	L18	Yayla305	Compatible	SecondLeaf	3	2	3	12	1
	39	0.043684646							
692	L18	Yayla305	Compatible	FirstLeaf	4	2	3	12	1
	35	0.319415042							
693	L18	Yayla305	Compatible	SecondLeaf	4	2	3	12	1
	35	0.220465745							
703	L18	Yayla305	Compatible	Crown 6	2	3	12	1	35
	1.00E-08								
705	L18	Yayla305	Compatible	SecondLeaf	6	2	3	12	1
	35	1.00E-08							
713	L18	Yayla305	Compatible	SecondLeaf	8	2	3	12	1
	35	0.190395962							
683	L18	Yayla305	Compatible	Crown 2	2	3	13	1	35
	0.069476168								
685	L18	Yayla305	Compatible	SecondLeaf	2	2	3	13	1
	35	0.550185827							
695	L18	Yayla305	Compatible	Crown 5	2	3	13	1	35
	1.00E-08								
697	L18	Yayla305	Compatible	SecondLeaf	5	2	3	13	1
	35	1.00E-08							
529	T34	HeinesVII	Compatible	Coleoptile	1	2	1	7	0
	14	0.059383821							
531	T34	HeinesVII	Compatible	Coleoptile	2	2	1	7	0
	12	0.296948772							

533	T34	HeinesVII	Compatible	Coleoptile	3	2	1	7	0
	13	0.01356778							
535	T34	HeinesVII	Compatible	Coleoptile	4	2	1	7	0
	14	0.132304013							
537	T34	HeinesVII	Compatible	Coleoptile	5	2	1	7	0
	12	0.242317238							
541	T34	HeinesVII	Compatible	Coleoptile	7	2	1	7	0
	13	0.451302827							
543	T34	HeinesVII	Compatible	Coleoptile	8	2	1	7	0
	14	0.038858084							
545	T34	HeinesVII	Compatible	Coleoptile	9	2	1	7	0
	13	0.116740075							
629	T34	HeinesVII	Compatible	Coleoptile	9	2	2	10	1
	20	0.249337129							
613	T34	HeinesVII	Compatible	Coleoptile	1	2	2	11	1
	20	1.00E-08							
615	T34	HeinesVII	Compatible	Coleoptile	2	2	2	11	1
	20	1.00E-08							
617	T34	HeinesVII	Compatible	Coleoptile	3	2	2	11	1
	20	0.037280165							
619	T34	HeinesVII	Compatible	Coleoptile	4	2	2	11	1
	20	0.17580787							
621	T34	HeinesVII	Compatible	Coleoptile	5	2	2	11	1
	20	0.060793275							
623	T34	HeinesVII	Compatible	Coleoptile	6	2	2	11	1
	20	1.00E-08							
625	T34	HeinesVII	Compatible	Coleoptile	7	2	2	11	1
	20	0.187053073							
627	T34	HeinesVII	Compatible	Coleoptile	8	2	2	11	1
	20	0.099151231							

807	T34	HeinesVII	Compatible	Crown 3	2	3	12	1	39
	0.036256981								
808	T34	HeinesVII	Compatible	FirstLeaf	3	2	3	12	1
	39	1.00E-08							
809	T34	HeinesVII	Compatible	SecondLeaf	3	2	3	12	1
	39	1.00E-08							
811	T34	HeinesVII	Compatible	Crown 4	2	3	12	1	35
	0.021129764								
812	T34	HeinesVII	Compatible	FirstLeaf	4	2	3	12	1
	35	1.00E-08							
813	T34	HeinesVII	Compatible	SecondLeaf	4	2	3	12	1
	35	1.00E-08							
823	T34	HeinesVII	Compatible	Crown 7	2	3	12	1	35
	0.008142121								
824	T34	HeinesVII	Compatible	FirstLeaf	7	2	3	12	1
	35	0.016137344							
825	T34	HeinesVII	Compatible	SecondLeaf	7	2	3	12	1
	35	1.00E-08							
831	T34	HeinesVII	Compatible	Crown 9	2	3	12	1	35
	0.037413732								
832	T34	HeinesVII	Compatible	FirstLeaf	9	2	3	12	1
	35	0.156506235							
833	T34	HeinesVII	Compatible	SecondLeaf	9	2	3	12	1
	35	0.009075284							
799	T34	HeinesVII	Compatible	Crown 1	2	3	13	1	35
	0.003300984								
800	T34	HeinesVII	Compatible	FirstLeaf	1	2	3	13	1
	35	1.00E-08							
801	T34	HeinesVII	Compatible	SecondLeaf	1	2	3	13	1
	35	1.00E-08							

803	T34	HeinesVII	Compatible	Crown 2	2	3	13	1	35
	1.00E-08								
804	T34	HeinesVII	Compatible	FirstLeaf	2	2	3	13	1
	35	1.00E-08							
805	T34	HeinesVII	Compatible	SecondLeaf	2	2	3	13	1
	35	1.00E-08							
815	T34	HeinesVII	Compatible	Crown 5	2	3	13	1	35
	1.00E-08								
816	T34	HeinesVII	Compatible	FirstLeaf	5	2	3	13	1
	35	1.00E-08							
817	T34	HeinesVII	Compatible	SecondLeaf	5	2	3	13	1
	35	1.00E-08							
819	T34	HeinesVII	Compatible	Crown 6	2	3	13	1	35
	0.005047012								
820	T34	HeinesVII	Compatible	FirstLeaf	6	2	3	13	1
	35	1.00E-08							
821	T34	HeinesVII	Compatible	SecondLeaf	6	2	3	13	1
	35	1.00E-08							
1233	T34	HeinesVII	Compatible	Crown 2	2	4	29	3	
	150	1.00E-08							
1234	T34	HeinesVII	Compatible	FirstLeaf	2	2	4	29	3
	150	1.00E-08							
1235	T34	HeinesVII	Compatible	FirstNode	2	2	4	29	3
	150	1.00E-08							
1236	T34	HeinesVII	Compatible	SecondNode	2	2	4	29	3
	150	1.00E-08							
1237	T34	HeinesVII	Compatible	Devhead	2	2	4	29	3
	150	1.00E-08							
1238	T34	HeinesVII	Compatible	FirstNode	2	2	4	29	3
	150	1.00E-08							

1239	T34	HeinesVII	Compatible	FirstLeaf	2	2	4	29	3
	150	1.00E-08							
1240	T34	HeinesVII	Compatible	SecondNode	2	2	4	29	3
	150	1.00E-08							
1241	T34	HeinesVII	Compatible	Devhead	2	2	4	29	3
	150	1.00E-08							
1242	T34	HeinesVII	Compatible	FirstNode	2	2	4	29	3
	150	1.00E-08							
1243	T34	HeinesVII	Compatible	FirstLeaf	2	2	4	29	3
	150	1.00E-08							
1244	T34	HeinesVII	Compatible	SecondNode	2	2	4	29	3
	150	1.00E-08							
1245	T34	HeinesVII	Compatible	Devhead	2	2	4	29	3
	150	1.00E-08							
1186	T34	HeinesVII	Compatible	Crown 1	2	5	33	7	
	148	1.00E-08							
1187	T34	HeinesVII	Compatible	FirstNode	1	2	5	33	7
	148	1.00E-08							
1188	T34	HeinesVII	Compatible	SecondNode	1	2	5	33	7
	148	1.00E-08							
1189	T34	HeinesVII	Compatible	FirstLeaf	1	2	5	33	7
	148	1.00E-08							
1190	T34	HeinesVII	Compatible	ThirdNode	1	2	5	33	7
	148	1.00E-08							
1191	T34	HeinesVII	Compatible	FourthNode	1	2	5	33	7
	148	1.00E-08							
1192	T34	HeinesVII	Compatible	Stem 1	2	5	33	7	
	148	1.00E-08							
1193	T34	HeinesVII	Compatible	FirstNode	1	2	5	33	7
	148	1.00E-08							

1194	T34	HeinesVII	Compatible	SecondNode	1	2	5	33	7
	148	1.00E-08							
1195	T34	HeinesVII	Compatible	FirstLeaf	1	2	5	33	7
	148	1.00E-08							
1196	T34	HeinesVII	Compatible	ThirdNode	1	2	5	33	7
	148	1.00E-08							
1197	T34	HeinesVII	Compatible	Devhead	1	2	5	33	7
	148	1.00E-08							
1198	T34	HeinesVII	Compatible	FirstNode	1	2	5	33	7
	148	1.00E-08							
1199	T34	HeinesVII	Compatible	SecondNode	1	2	5	33	7
	148	1.00E-08							
1200	T34	HeinesVII	Compatible	ThirdNode	1	2	5	33	7
	148	1.00E-08							
1201	T34	HeinesVII	Compatible	Devhead	1	2	5	33	7
	148	1.00E-08							
565	T34	Yayla305	Incompatible	Coleoptile	1	2	1	7	0
	14	0.096551816							
567	T34	Yayla305	Incompatible	Coleoptile	2	2	1	7	0
	12	0.094652303							
575	T34	Yayla305	Incompatible	Coleoptile	6	2	1	7	0
	14	0.040267804							
579	T34	Yayla305	Incompatible	Coleoptile	8	2	1	7	0
	13	0.125730024							
581	T34	Yayla305	Incompatible	Coleoptile	9	2	1	7	0
	13	0.299452758							
573	T34	Yayla305	Incompatible	Coleoptile	5	2	1	9	0
	14	0.093401749							
661	T34	Yayla305	Incompatible	Coleoptile	7	2	2	10	1
	20	0.089125392							

649	T34	Yayla305	Incompatible	Coleoptile	1	2	2	11	1	
	19	0.006013583								
653	T34	Yayla305	Incompatible	Coleoptile	3	2	2	11	1	
	20	0.037006998								
655	T34	Yayla305	Incompatible	Coleoptile	4	2	2	11	1	
	20	0.002325113								
657	T34	Yayla305	Incompatible	Coleoptile	5	2	2	11	1	
	19	0.001616459								
659	T34	Yayla305	Incompatible	Coleoptile	6	2	2	11	1	
	19	0.028106572								
663	T34	Yayla305	Incompatible	Coleoptile	8	2	2	11	1	
	20	0.024177003								
665	T34	Yayla305	Incompatible	Coleoptile	9	2	2	11	1	
	20	0.000519619								
763	T34	Yayla305	Incompatible	Crown	1	2	3	12	1	35
	0.030817286									
764	T34	Yayla305	Incompatible	FirstLeaf	1	2	3	12	1	
	35	0.007697694								
765	T34	Yayla305	Incompatible	SecondLeaf	1	2	3	12	1	
	35	1.00E-08								
771	T34	Yayla305	Incompatible	Crown	3	2	3	12	1	39
	0.010082825									
772	T34	Yayla305	Incompatible	FirstLeaf	3	2	3	12	1	
	39	0.003967696								
773	T34	Yayla305	Incompatible	SecondLeaf	3	2	3	12	1	
	39	0.001005143								
775	T34	Yayla305	Incompatible	Crown	4	2	3	12	1	35
	0.021160662									
776	T34	Yayla305	Incompatible	FirstLeaf	4	2	3	12	1	
	35	1.00E-08								

777	T34	Yayla305	Incompatible	SecondLeaf	4	2	3	12	1	35
0.004930461										
787	T34	Yayla305	Incompatible	Crown	6	2	3	12	1	35
0.122157323										
788	T34	Yayla305	Incompatible	FirstLeaf	6	2	3	12	1	35
0.044951193										
789	T34	Yayla305	Incompatible	SecondLeaf	6	2	3	12	1	35
1.00E-08										
791	T34	Yayla305	Incompatible	Crown	7	2	3	12	1	35
1.00E-08										
792	T34	Yayla305	Incompatible	FirstLeaf	7	2	3	12	1	35
1.00E-08										
793	T34	Yayla305	Incompatible	SecondLeaf	7	2	3	12	1	35
1.00E-08										
795	T34	Yayla305	Incompatible	Crown	8	2	3	12	1	35
0.018861										
796	T34	Yayla305	Incompatible	FirstLeaf	8	2	3	12	1	35
0.223825354										
797	T34	Yayla305	Incompatible	SecondLeaf	8	2	3	12	1	35
0.062299687										
768	T34	Yayla305	Incompatible	FirstLeaf	2	2	3	13	1	35
1.00E-08										
769	T34	Yayla305	Incompatible	SecondLeaf	2	2	3	13	1	35
1.00E-08										
779	T34	Yayla305	Incompatible	Crown	5	2	3	13	1	35
0.016065198										
781	T34	Yayla305	Incompatible	SecondLeaf	5	2	3	13	1	35
1.00E-08										
1265	T34	Yayla305	Incompatible	Crown	1	2	4	26	3	151
1.00E-08										

1266	T34	Yayla305	Incompatible	FirstNode	1	2	4	26	3
	151	1.00E-08							
1267	T34	Yayla305	Incompatible	SecondNode	1	2	4	26	3
	151	1.00E-08							
1268	T34	Yayla305	Incompatible	FirstLeaf	1	2	4	26	3
	151	1.00E-08							
1269	T34	Yayla305	Incompatible	ThirdNode	1	2	4	26	3
	151	1.00E-08							
1270	T34	Yayla305	Incompatible	Devhead	1	2	4	26	3
	151	1.00E-08							
1271	T34	Yayla305	Incompatible	FirstNode	1	2	4	26	3
	151	1.00E-08							
1274	T34	Yayla305	Incompatible	Devhead	1	2	4	26	3
	151	1.00E-08							
1276	T34	Yayla305	Incompatible	SecondNode	1	2	4	26	3
	151	1.00E-08							
1277	T34	Yayla305	Incompatible	Devhead	1	2	4	26	3
	151	1.00E-08							
1297	T34	Yayla305	Incompatible	FirstNode	2	2	5	33	7
	151	1.00E-08							
1298	T34	Yayla305	Incompatible	SecondNode	2	2	5	33	7
	151	1.00E-08							
1299	T34	Yayla305	Incompatible	FirstLeaf	2	2	5	33	7
	151	1.00E-08							
1300	T34	Yayla305	Incompatible	ThirdNode	2	2	5	33	7
	151	1.00E-08							
1301	T34	Yayla305	Incompatible	FourthNode	2	2	5	33	7
	151	1.00E-08							
1302	T34	Yayla305	Incompatible	Devhead	2	2	5	33	7
	151	1.00E-08							

847	L18	HeinesVII	Compatible	Coleoptile	1	3	1	7	0
	15	1.00E-08							
849	L18	HeinesVII	Compatible	Coleoptile	2	3	1	7	0
	15	1.00E-08							
851	L18	HeinesVII	Compatible	Coleoptile	3	3	1	7	0
	15	0.068134786							
853	L18	HeinesVII	Compatible	Coleoptile	4	3	1	7	0
	15	1.00E-08							
855	L18	HeinesVII	Compatible	Coleoptile	5	3	1	7	0
	15	0.019249004							
857	L18	HeinesVII	Compatible	Coleoptile	6	3	1	7	0
	15	0.015950343							
859	L18	HeinesVII	Compatible	Coleoptile	7	3	1	7	0
	15	1.00E-08							
861	L18	HeinesVII	Compatible	Coleoptile	8	3	1	7	0
	15	0.03860595							
863	L18	HeinesVII	Compatible	Coleoptile	9	3	1	7	0
	15	1.00E-08							
931	L18	HeinesVII	Compatible	Coleoptile	1	3	2	11	1
	21	1.00E-08							
933	L18	HeinesVII	Compatible	Coleoptile	2	3	2	11	1
	21	1.00E-08							
935	L18	HeinesVII	Compatible	Coleoptile	3	3	2	11	1
	21	1.00E-08							
937	L18	HeinesVII	Compatible	Coleoptile	4	3	2	11	1
	21	1.00E-08							
939	L18	HeinesVII	Compatible	Coleoptile	5	3	2	11	1
	21	1.00E-08							
943	L18	HeinesVII	Compatible	Coleoptile	6	3	2	11	1
	21	1.00E-08							

945	L18	HeinesVII	Compatible	Coleoptile	7	3	2	11	1
21	1.00E-08								
947	L18	HeinesVII	Compatible	Coleoptile	8	3	2	11	1
21	1.00E-08								
1015	L18	HeinesVII	Compatible	Crown 1	3	3	12	1	45
1.00E-08									
1016	L18	HeinesVII	Compatible	FirstLeaf	1	3	3	12	1
45	1.00E-08								
1017	L18	HeinesVII	Compatible	SecondLeaf	1	3	3	12	1
45	0.083616328								
1019	L18	HeinesVII	Compatible	Crown 2	3	3	12	1	41
1.00E-08									
1020	L18	HeinesVII	Compatible	FirstLeaf	2	3	3	12	1
41	1.00E-08								
1021	L18	HeinesVII	Compatible	SecondLeaf	2	3	3	12	1
41	1.00E-08								
1023	L18	HeinesVII	Compatible	Crown 3	3	3	12	1	39
1.00E-08									
1024	L18	HeinesVII	Compatible	FirstLeaf	3	3	3	12	1
39	1.00E-08								
1025	L18	HeinesVII	Compatible	SecondLeaf	3	3	3	12	1
39	1.00E-08								
1027	L18	HeinesVII	Compatible	Crown 4	3	3	12	1	42
0.016321597									
1028	L18	HeinesVII	Compatible	FirstLeaf	4	3	3	12	1
42	1.00E-08								
1029	L18	HeinesVII	Compatible	SecondLeaf	4	3	3	12	1
42	1.00E-08								
1031	L18	HeinesVII	Compatible	Crown 5	3	3	12	1	46
0.045214859									

1032	L18	HeinesVII	Compatible	FirstLeaf	5	3	3	12	1
	46	0.293106555							
1033	L18	HeinesVII	Compatible	SecondLeaf	5	3	3	12	1
	46	0.078947987							
1035	L18	HeinesVII	Compatible	Crown 6	3	3	12	1	40
	0.040763509								
1036	L18	HeinesVII	Compatible	FirstLeaf	6	3	3	12	1
	40	1.00E-08							
1039	L18	HeinesVII	Compatible	Crown 7	3	3	12	1	46
	0.250967465								
1041	L18	HeinesVII	Compatible	SecondLeaf	7	3	3	12	1
	46	0.074632892							
1043	L18	HeinesVII	Compatible	Crown 8	3	3	12	1	46
	0.139026233								
1044	L18	HeinesVII	Compatible	FirstLeaf	8	3	3	12	1
	46	0.33021966							
1045	L18	HeinesVII	Compatible	SecondLeaf	8	3	3	12	1
	46	0.54753793							
1047	L18	HeinesVII	Compatible	Crown 9	3	3	12	1	45
	1.00E-08								
1048	L18	HeinesVII	Compatible	FirstLeaf	9	3	3	12	1
	45	1.00E-08							
1049	L18	HeinesVII	Compatible	SecondLeaf	9	3	3	12	1
	45	1.00E-08							
1317	L18	HeinesVII	Compatible	FirstLeaf	1	3	4	32	7
	119	0.587235444							
1307	L18	HeinesVII	Compatible	Crown 1	3	5	32	7	
	119	1.00E-08							
1308	L18	HeinesVII	Compatible	FirstNode	1	3	5	32	7
	119	0.088021829							

1309	L18	HeinesVII	Compatible	FirstLeaf	1	3	5	32	7
	119	0.011241816							
1310	L18	HeinesVII	Compatible	SecondNode	1	3	5	32	7
	119	0.056020033							
1312	L18	HeinesVII	Compatible	FirstNode	1	3	5	32	7
	119	0.002556368							
1313	L18	HeinesVII	Compatible	SecondNode	1	3	5	32	7
	119	0.000590112							
1314	L18	HeinesVII	Compatible	Devhead	1	3	5	32	7
	119	0.076976291							
1315	L18	HeinesVII	Compatible	FirstNode	1	3	5	32	7
	119	0.274593458							
1316	L18	HeinesVII	Compatible	SecondNode	1	3	5	32	7
	119	0.178051892							
1318	L18	HeinesVII	Compatible	ThirdNode	1	3	5	32	7
	119	0.405804998							
1358	L18	HeinesVII	Compatible	Crown 2	3	5	32	7	
	121	1.00E-08							
1361	L18	HeinesVII	Compatible	SecondNode	2	3	5	32	7
	121	1.00E-08							
1362	L18	HeinesVII	Compatible	Devhead	2	3	5	32	7
	121	1.00E-08							
1365	L18	HeinesVII	Compatible	SecondNode	2	3	5	32	7
	121	1.00E-08							
1366	L18	HeinesVII	Compatible	Devhead	2	3	5	32	7
	121	1.00E-08							
1367	L18	HeinesVII	Compatible	FirstNode	2	3	5	32	7
	121	1.00E-08							
1368	L18	HeinesVII	Compatible	FirstLeaf	2	3	5	32	7
	121	1.00E-08							

1369	L18	HeinesVII	Compatible	SecondNode	2	3	5	32	7
	121	1.00E-08							
1370	L18	HeinesVII	Compatible	ThirdNode	2	3	5	32	7
	121	1.00E-08							
1371	L18	HeinesVII	Compatible	Devhead	2	3	5	32	7
	121	1.00E-08							
1453	L18	HeinesVII	Compatible	FirstNode	3	3	5	32	7
	123	1.00E-08							
1454	L18	HeinesVII	Compatible	SecondNode	3	3	5	32	7
	123	1.00E-08							
1455	L18	HeinesVII	Compatible	FirstLeaf	3	3	5	32	7
	123	1.00E-08							
1456	L18	HeinesVII	Compatible	Devhead	3	3	5	32	7
	123	1.00E-08							
1457	L18	HeinesVII	Compatible	FirstNode	3	3	5	32	7
	123	1.00E-08							
1458	L18	HeinesVII	Compatible	SecondNode	3	3	5	32	7
	123	1.00E-08							
1459	L18	HeinesVII	Compatible	FirstLeaf	3	3	5	32	7
	123	1.00E-08							
1460	L18	HeinesVII	Compatible	ThirdNode	3	3	5	32	7
	123	1.00E-08							
1461	L18	HeinesVII	Compatible	Devhead	3	3	5	32	7
	123	1.00E-08							
1462	L18	HeinesVII	Compatible	FirstNode	3	3	5	32	7
	123	1.00E-08							
1464	L18	HeinesVII	Compatible	SecondNode	3	3	5	32	7
	123	1.00E-08							
1465	L18	HeinesVII	Compatible	Devhead	3	3	5	32	7
	123	1.00E-08							

1543	L18	HeinesVII	Compatible	Crown	5	3	5	33	7
	125	0.063434891							
1544	L18	HeinesVII	Compatible	FirstNode	5	3	5	33	7
	125	0.048895476							
1546	L18	HeinesVII	Compatible	SecondNode	5	3	5	33	7
	125	1.00E-08							
1547	L18	HeinesVII	Compatible	ThirdNode	5	3	5	33	7
	125	1.00E-08							
1548	L18	HeinesVII	Compatible	FourthNode	5	3	5	33	7
	125	1.00E-08							
1549	L18	HeinesVII	Compatible	Devhead	5	3	5	33	7
	125	1.00E-08							
1550	L18	HeinesVII	Compatible	FirstNode	5	3	5	33	7
	125	0.077937582							
1551	L18	HeinesVII	Compatible	SecondNode	5	3	5	33	7
	125	1.00E-08							
1553	L18	HeinesVII	Compatible	Devhead	5	3	5	33	7
	125	1.00E-08							
1554	L18	HeinesVII	Compatible	FirstNode	5	3	5	33	7
	125	1.00E-08							
1555	L18	HeinesVII	Compatible	SecondNode	5	3	5	33	7
	125	1.00E-08							
1557	L18	HeinesVII	Compatible	ThirdNode	5	3	5	33	7
	125	1.00E-08							
1558	L18	HeinesVII	Compatible	Devhead	5	3	5	33	7
	125	1.00E-08							
1499	L18	HeinesVII	Compatible	FirstNode	4	3	5	34	7
	124	1.00E-08							
1500	L18	HeinesVII	Compatible	FirstLeaf	4	3	5	34	7
	124	1.00E-08							

1501	L18	HeinesVII	Compatible	SecondNode	4	3	5	34	7
	124	1.00E-08							
1502	L18	HeinesVII	Compatible	Devhead	4	3	5	34	7
	124	1.00E-08							
1503	L18	HeinesVII	Compatible	FirstNode	4	3	5	34	7
	124	1.00E-08							
1504	L18	HeinesVII	Compatible	FirstLeaf	4	3	5	34	7
	124	1.00E-08							
1505	L18	HeinesVII	Compatible	SecondNode	4	3	5	34	7
	124	1.00E-08							
1506	L18	HeinesVII	Compatible	ThirdNode	4	3	5	34	7
	124	1.00E-08							
1507	L18	HeinesVII	Compatible	Devhead	4	3	5	34	7
	124	1.00E-08							
1508	L18	HeinesVII	Compatible	FirstNode	4	3	5	34	7
	124	1.00E-08							
1510	L18	HeinesVII	Compatible	SecondNode	4	3	5	34	7
	124	1.00E-08							
1511	L18	HeinesVII	Compatible	Devhead	4	3	5	34	7
	124	1.00E-08							
883	L18	Yayla305	Compatible	Coleoptile	1	3	1	7	0
	15	0.011067161							
885	L18	Yayla305	Compatible	Coleoptile	2	3	1	7	0
	15	0.013820784							
889	L18	Yayla305	Compatible	Coleoptile	4	3	1	7	0
	15	0.024226568							
891	L18	Yayla305	Compatible	Coleoptile	5	3	1	7	0
	15	1.00E-08							
893	L18	Yayla305	Compatible	Coleoptile	6	3	1	7	0
	15	0.050747102							

895	L18	Yayla305	Compatible	Coleoptile	7	3	1	7	0
	15	0.121081094							
897	L18	Yayla305	Compatible	Coleoptile	8	3	1	7	0
	15	0.017309792							
899	L18	Yayla305	Compatible	Coleoptile	9	3	1	7	0
	15	1.00E-08							
967	L18	Yayla305	Compatible	Coleoptile	1	3	2	11	1
	21	1.00E-08							
969	L18	Yayla305	Compatible	Coleoptile	2	3	2	11	1
	21	1.00E-08							
971	L18	Yayla305	Compatible	Coleoptile	3	3	2	11	1
	21	1.00E-08							
973	L18	Yayla305	Compatible	Coleoptile	4	3	2	11	1
	21	1.00E-08							
977	L18	Yayla305	Compatible	Coleoptile	5	3	2	11	1
	21	1.00E-08							
979	L18	Yayla305	Compatible	Coleoptile	6	3	2	11	1
	21	1.00E-08							
983	L18	Yayla305	Compatible	Coleoptile	7	3	2	11	1
	21	1.00E-08							
1087	L18	Yayla305	Compatible	Crown 1	3	3	12	1	39
	1.00E-08								
1088	L18	Yayla305	Compatible	FirstLeaf	1	3	3	12	1
	39	0.000535049							
1089	L18	Yayla305	Compatible	SecondLeaf	1	3	3	12	1
	39	1.00E-08							
1091	L18	Yayla305	Compatible	Crown 2	3	3	12	1	41
	0.018177388								
1093	L18	Yayla305	Compatible	SecondLeaf	2	3	3	12	1
	41	0.007551106							

1095	L18	Yayla305	Compatible	Crown 3	3	3	12	1	38
	0.003409942								
1096	L18	Yayla305	Compatible	FirstLeaf	3	3	3	12	1
	38	0.014544401							
1097	L18	Yayla305	Compatible	SecondLeaf	3	3	3	12	1
	38	1.00E-08							
1099	L18	Yayla305	Compatible	Crown 4	3	3	12	1	46
	1.00E-08								
1100	L18	Yayla305	Compatible	FirstLeaf	4	3	3	12	1
	46	0.371627736							
1101	L18	Yayla305	Compatible	SecondLeaf	4	3	3	12	1
	46	0.136030561							
1103	L18	Yayla305	Compatible	Crown 5	3	3	12	1	46
	0.013878232								
1104	L18	Yayla305	Compatible	FirstLeaf	5	3	3	12	1
	46	0.095493267							
1105	L18	Yayla305	Compatible	SecondLeaf	5	3	3	12	1
	46	0.001780056							
1107	L18	Yayla305	Compatible	Crown 6	3	3	12	1	45
	0.087557629								
1108	L18	Yayla305	Compatible	FirstLeaf	6	3	3	12	1
	45	0.185090831							
1109	L18	Yayla305	Compatible	SecondLeaf	6	3	3	12	1
	45	0.111190964							
1111	L18	Yayla305	Compatible	Crown 7	3	3	12	1	42
	0.002204519								
1112	L18	Yayla305	Compatible	FirstLeaf	7	3	3	12	1
	42	1.00E-08							
1113	L18	Yayla305	Compatible	SecondLeaf	7	3	3	12	1
	42	1.00E-08							

1115	L18	Yayla305	Compatible	Crown 8	3	3	12	1	42
	0.002632176								
1116	L18	Yayla305	Compatible	FirstLeaf	8	3	3	12	1
	42	0.002484043							
1117	L18	Yayla305	Compatible	SecondLeaf	8	3	3	12	1
	42	1.00E-08							
1119	L18	Yayla305	Compatible	Crown 9	3	3	12	1	45
	0.001475311								
1120	L18	Yayla305	Compatible	FirstLeaf	9	3	3	12	1
	45	1.00E-08							
1121	L18	Yayla305	Compatible	SecondLeaf	9	3	3	12	1
	45	1.00E-08							
1372	L18	Yayla305	Compatible	Crown 2	3	4	31	6	
	122	0.184032842							
1376	L18	Yayla305	Compatible	Devhead	2	3	4	31	6
	122	0.27902594							
1384	L18	Yayla305	Compatible	Devhead	2	3	4	31	6
	122	0.251420829							
1345	L18	Yayla305	Compatible	FirstNode	1	3	5	32	7
	121	1.00E-08							
1346	L18	Yayla305	Compatible	FirstLeaf	1	3	5	32	7
	121	1.00E-08							
1347	L18	Yayla305	Compatible	SecondNode	1	3	5	32	7
	121	1.00E-08							
1348	L18	Yayla305	Compatible	Devhead	1	3	5	32	7
	121	1.00E-08							
1435	L18	Yayla305	Compatible	Crown 3	3	5	32	7	
	123	1.00E-08							
1436	L18	Yayla305	Compatible	FirstNode	3	3	5	32	7
	123	1.00E-08							

1437	L18	Yayla305	Compatible	SecondNode	3	3	5	32	7
	123	1.00E-08							
1439	L18	Yayla305	Compatible	ThirdNode	3	3	5	32	7
	123	1.00E-08							
1440	L18	Yayla305	Compatible	Devhead	3	3	5	32	7
	123	1.00E-08							
1441	L18	Yayla305	Compatible	FirstNode	3	3	5	32	7
	123	0.146332557							
1442	L18	Yayla305	Compatible	SecondNode	3	3	5	32	7
	123	0.182793551							
1443	L18	Yayla305	Compatible	FirstLeaf	3	3	5	32	7
	123	0.375337439							
1444	L18	Yayla305	Compatible	ThirdNode	3	3	5	32	7
	123	0.223116325							
1445	L18	Yayla305	Compatible	Devhead	3	3	5	32	7
	123	0.222281613							
1448	L18	Yayla305	Compatible	SecondNode	3	3	5	32	7
	123	0.462090166							
1449	L18	Yayla305	Compatible	FirstLeaf	3	3	5	32	7
	123	0.27332298							
1450	L18	Yayla305	Compatible	ThirdNode	3	3	5	32	7
	123	0.168372798							
1451	L18	Yayla305	Compatible	Devhead	3	3	5	32	7
	123	0.21991116							
1344	L18	Yayla305	Compatible	Crown 1	3	5	33	7	
	121	1.00E-08							
1349	L18	Yayla305	Compatible	FirstNode	1	3	5	33	7
	121	1.00E-08							
1350	L18	Yayla305	Compatible	SecondNode	1	3	5	33	7
	121	1.00E-08							

1351	L18	Yayla305	Compatible	FirstLeaf	1	3	5	33	7
	121	1.00E-08							
1352	L18	Yayla305	Compatible	ThirdNode	1	3	5	33	7
	121	1.00E-08							
1353	L18	Yayla305	Compatible	Devhead	1	3	5	33	7
	121	1.00E-08							
1355	L18	Yayla305	Compatible	FirstLeaf	1	3	5	33	7
	121	1.00E-08							
1356	L18	Yayla305	Compatible	SecondNode	1	3	5	33	7
	121	1.00E-08							
1357	L18	Yayla305	Compatible	Devhead	1	3	5	33	7
	121	1.00E-08							
1485	L18	Yayla305	Compatible	FirstNode	4	3	5	33	7
	123	1.00E-08							
1487	L18	Yayla305	Compatible	SecondNode	4	3	5	33	7
	123	1.00E-08							
1488	L18	Yayla305	Compatible	Devhead	4	3	5	33	7
	123	1.00E-08							
1489	L18	Yayla305	Compatible	FirstNode	4	3	5	33	7
	123	1.00E-08							
1490	L18	Yayla305	Compatible	SecondNode	4	3	5	33	7
	123	0.001206534							
1491	L18	Yayla305	Compatible	FirstLeaf	4	3	5	33	7
	123	1.00E-08							
1492	L18	Yayla305	Compatible	Devhead	4	3	5	33	7
	123	1.00E-08							
1493	L18	Yayla305	Compatible	FirstNode	4	3	5	33	7
	123	0.063698786							
1494	L18	Yayla305	Compatible	SecondNode	4	3	5	33	7
	123	0.134039326							

1496	L18	Yayla305	Compatible	ThirdNode	4	3	5	33	7
	123	0.123359227							
1528	L18	Yayla305	Compatible	FirstNode	5	3	5	33	7
	124	0.032195804							
1529	L18	Yayla305	Compatible	SecondNode	5	3	5	33	7
	124	0.043562712							
1531	L18	Yayla305	Compatible	ThirdNode	5	3	5	33	7
	124	1.00E-08							
1532	L18	Yayla305	Compatible	Devhead	5	3	5	33	7
	124	0.050371303							
1537	L18	Yayla305	Compatible	FirstNode	5	3	5	33	7
	124	0.09693178							
1539	L18	Yayla305	Compatible	FirstLeaf	5	3	5	33	7
	124	0.575869636							
1542	L18	Yayla305	Compatible	Devhead	5	3	5	33	7
	124	0.089287739							
865	T34	HeinesVII	Compatible	Coleoptile	1	3	1	7	0
	15	0.083453683							
867	T34	HeinesVII	Compatible	Coleoptile	2	3	1	7	0
	15	0.049310981							
869	T34	HeinesVII	Compatible	Coleoptile	3	3	1	7	0
	15	0.122468591							
871	T34	HeinesVII	Compatible	Coleoptile	4	3	1	7	0
	15	0.185380846							
873	T34	HeinesVII	Compatible	Coleoptile	5	3	1	7	0
	15	0.06832985							
875	T34	HeinesVII	Compatible	Coleoptile	6	3	1	7	0
	15	0.085078525							
877	T34	HeinesVII	Compatible	Coleoptile	7	3	1	7	0
	15	0.090669716							

879	T34	HeinesVII	Compatible	Coleoptile	8	3	1	7	0
	15	0.044522876							
881	T34	HeinesVII	Compatible	Coleoptile	9	3	1	7	0
	15	0.062027669							
949	T34	HeinesVII	Compatible	Coleoptile	1	3	2	11	1
	21	1.00E-08							
951	T34	HeinesVII	Compatible	Coleoptile	2	3	2	11	1
	21	1.00E-08							
953	T34	HeinesVII	Compatible	Coleoptile	3	3	2	11	1
	21	1.00E-08							
955	T34	HeinesVII	Compatible	Coleoptile	4	3	2	11	1
	21	1.00E-08							
957	T34	HeinesVII	Compatible	Coleoptile	5	3	2	11	1
	21	1.00E-08							
959	T34	HeinesVII	Compatible	Coleoptile	6	3	2	11	1
	21	1.00E-08							
961	T34	HeinesVII	Compatible	Coleoptile	7	3	2	11	1
	21	1.00E-08							
963	T34	HeinesVII	Compatible	Coleoptile	8	3	2	11	1
	21	0.013517374							
965	T34	HeinesVII	Compatible	Coleoptile	9	3	2	11	1
	21	1.00E-08							
1051	T34	HeinesVII	Compatible	Crown 1	3	3	12	1	38
	1.00E-08								
1052	T34	HeinesVII	Compatible	FirstLeaf	1	3	3	12	1
	38	0.151316431							
1053	T34	HeinesVII	Compatible	SecondLeaf	1	3	3	12	1
	38	1.00E-08							
1055	T34	HeinesVII	Compatible	Crown 2	3	3	12	1	41
	1.00E-08								

1056	T34	HeinesVII	Compatible	FirstLeaf	2	3	3	12	1
	41	1.00E-08							
1057	T34	HeinesVII	Compatible	SecondLeaf	2	3	3	12	1
	41	1.00E-08							
1059	T34	HeinesVII	Compatible	Crown 3	3	3	12	1	40
	1.00E-08								
1060	T34	HeinesVII	Compatible	FirstLeaf	3	3	3	12	1
	40	1.00E-08							
1061	T34	HeinesVII	Compatible	SecondLeaf	3	3	3	12	1
	40	1.00E-08							
1063	T34	HeinesVII	Compatible	Crown 4	3	3	12	1	42
	1.00E-08								
1064	T34	HeinesVII	Compatible	FirstLeaf	4	3	3	12	1
	42	1.00E-08							
1065	T34	HeinesVII	Compatible	SecondLeaf	4	3	3	12	1
	42	1.00E-08							
1067	T34	HeinesVII	Compatible	Crown 5	3	3	12	1	46
	0.49131082								
1068	T34	HeinesVII	Compatible	FirstLeaf	5	3	3	12	1
	46	0.471060792							
1069	T34	HeinesVII	Compatible	SecondLeaf	5	3	3	12	1
	46	0.252044553							
1071	T34	HeinesVII	Compatible	Crown 6	3	3	12	1	46
	1.00E-08								
1072	T34	HeinesVII	Compatible	FirstLeaf	6	3	3	12	1
	46	1.00E-08							
1073	T34	HeinesVII	Compatible	SecondLeaf	6	3	3	12	1
	46	1.00E-08							
1075	T34	HeinesVII	Compatible	Crown 7	3	3	12	1	46
	0.278740403								

1076	T34	HeinesVII	Compatible	FirstLeaf	7	3	3	12	1
	46	0.018079295							
1077	T34	HeinesVII	Compatible	SecondLeaf	7	3	3	12	1
	46	0.535631674							
1080	T34	HeinesVII	Compatible	FirstLeaf	8	3	3	12	1
	42	0.021153785							
1081	T34	HeinesVII	Compatible	SecondLeaf	8	3	3	12	1
	42	0.016922446							
1083	T34	HeinesVII	Compatible	Crown 9	3	3	12	1	45
	0.128921175								
1084	T34	HeinesVII	Compatible	FirstLeaf	9	3	3	12	1
	45	0.283281149							
1085	T34	HeinesVII	Compatible	SecondLeaf	9	3	3	12	1
	45	0.137068467							
1303	T34	HeinesVII	Compatible	Crown 1	3	4	15	4	
	119	1.00E-08							
1304	T34	HeinesVII	Compatible	FirstNode	1	3	4	15	4
	119	1.00E-08							
1305	T34	HeinesVII	Compatible	FirstLeaf	1	3	4	15	4
	119	0.003191558							
1306	T34	HeinesVII	Compatible	SecondNode	1	3	4	15	4
	119	1.00E-08							
1424	T34	HeinesVII	Compatible	FirstNode	3	3	4	31	6
	123	1.00E-08							
1425	T34	HeinesVII	Compatible	FirstLeaf	3	3	4	31	6
	123	1.00E-08							
1426	T34	HeinesVII	Compatible	SecondNode	3	3	4	31	6
	123	1.00E-08							
1427	T34	HeinesVII	Compatible	Devhead	3	3	4	31	6
	123	1.00E-08							

1428	T34	HeinesVII	Compatible	FirstNode	3	3	4	31	6
	123	1.00E-08							
1429	T34	HeinesVII	Compatible	FirstLeaf	3	3	4	31	6
	123	1.00E-08							
1430	T34	HeinesVII	Compatible	SecondNode	3	3	4	31	6
	123	1.00E-08							
1431	T34	HeinesVII	Compatible	Devhead	3	3	4	31	6
	123	1.00E-08							
1432	T34	HeinesVII	Compatible	FirstNode	3	3	4	31	6
	123	1.00E-08							
1433	T34	HeinesVII	Compatible	FirstLeaf	3	3	4	31	6
	123	1.00E-08							
1434	T34	HeinesVII	Compatible	Devhead	3	3	4	31	6
	123	1.00E-08							
1385	T34	HeinesVII	Compatible	Crown 2	3	5	32	7	
	122	1.00E-08							
1386	T34	HeinesVII	Compatible	FirstNode	2	3	5	32	7
	122	1.00E-08							
1387	T34	HeinesVII	Compatible	FirstLeaf	2	3	5	32	7
	122	1.00E-08							
1388	T34	HeinesVII	Compatible	SecondNode	2	3	5	32	7
	122	1.00E-08							
1389	T34	HeinesVII	Compatible	Devhead	2	3	5	32	7
	122	1.00E-08							
1390	T34	HeinesVII	Compatible	FirstNode	2	3	5	32	7
	122	1.00E-08							
1392	T34	HeinesVII	Compatible	SecondNode	2	3	5	32	7
	122	1.00E-08							
1393	T34	HeinesVII	Compatible	ThirdNode	2	3	5	32	7
	122	1.00E-08							

1394	T34	HeinesVII	Compatible	Devhead	2	3	5	32	7
	122	1.00E-08							
1395	T34	HeinesVII	Compatible	FirstNode	2	3	5	32	7
	122	1.00E-08							
1396	T34	HeinesVII	Compatible	FirstLeaf	2	3	5	32	7
	122	1.00E-08							
1397	T34	HeinesVII	Compatible	SecondNode	2	3	5	32	7
	122	1.00E-08							
1398	T34	HeinesVII	Compatible	Devhead	2	3	5	32	7
	122	1.00E-08							
1513	T34	HeinesVII	Compatible	FirstNode	4	3	5	32	7
	124	0.012947892							
1514	T34	HeinesVII	Compatible	FirstLeaf	4	3	5	32	7
	124	1.00E-08							
1515	T34	HeinesVII	Compatible	FirstLeaf	4	3	5	32	7
	124	1.00E-08							
1516	T34	HeinesVII	Compatible	SecondNode	4	3	5	32	7
	124	1.00E-08							
1517	T34	HeinesVII	Compatible	Devhead	4	3	5	32	7
	124	1.00E-08							
1518	T34	HeinesVII	Compatible	FirstNode	4	3	5	32	7
	124	1.00E-08							
1519	T34	HeinesVII	Compatible	FirstLeaf	4	3	5	32	7
	124	1.00E-08							
1520	T34	HeinesVII	Compatible	SecondNode	4	3	5	32	7
	124	1.00E-08							
1521	T34	HeinesVII	Compatible	ThirdNode	4	3	5	32	7
	124	1.00E-08							
1522	T34	HeinesVII	Compatible	Devhead	4	3	5	32	7
	124	1.00E-08							

1523	T34	HeinesVII	Compatible	FirstNode	4	3	5	32	7
	124	1.00E-08							
1525	T34	HeinesVII	Compatible	SecondNode	4	3	5	32	7
	124	1.00E-08							
1526	T34	HeinesVII	Compatible	Devhead	4	3	5	32	7
	124	1.00E-08							
1573	T34	HeinesVII	Compatible	Crown	5	3	5	32	7
	125	1.00E-08							
1574	T34	HeinesVII	Compatible	FirstNode	5	3	5	32	7
	125	1.00E-08							
1575	T34	HeinesVII	Compatible	FirstLeaf	5	3	5	32	7
	125	1.00E-08							
1576	T34	HeinesVII	Compatible	SecondNode	5	3	5	32	7
	125	1.00E-08							
1577	T34	HeinesVII	Compatible	ThirdNode	5	3	5	32	7
	125	1.00E-08							
1578	T34	HeinesVII	Compatible	Devhead	5	3	5	32	7
	125	1.00E-08							
1579	T34	HeinesVII	Compatible	FirstNode	5	3	5	32	7
	125	1.00E-08							
1581	T34	HeinesVII	Compatible	SecondNode	5	3	5	32	7
	125	1.00E-08							
1582	T34	HeinesVII	Compatible	ThirdNode	5	3	5	32	7
	125	1.00E-08							
1583	T34	HeinesVII	Compatible	Devhead	5	3	5	32	7
	125	1.00E-08							
1584	T34	HeinesVII	Compatible	FirstNode	5	3	5	32	7
	125	1.00E-08							
1586	T34	HeinesVII	Compatible	SecondNode	5	3	5	32	7
	125	1.00E-08							

1587	T34	HeinesVII	Compatible	Devhead	5	3	5	32	7
	125	1.00E-08							
901	T34	Yayla305	Incompatible	Coleoptile	1	3	1	7	0
	15	0.019568056							
903	T34	Yayla305	Incompatible	Coleoptile	2	3	1	7	0
	15	0.033189357							
905	T34	Yayla305	Incompatible	Coleoptile	3	3	1	7	0
	15	0.231690754							
907	T34	Yayla305	Incompatible	Coleoptile	4	3	1	7	0
	15	0.090348724							
911	T34	Yayla305	Incompatible	Coleoptile	6	3	1	7	0
	15	0.088603371							
913	T34	Yayla305	Incompatible	Coleoptile	7	3	1	7	0
	15	0.070087945							
915	T34	Yayla305	Incompatible	Coleoptile	8	3	1	7	0
	15	0.219790419							
917	T34	Yayla305	Incompatible	Coleoptile	9	3	1	7	0
	15	0.229794619							
985	T34	Yayla305	Incompatible	Coleoptile	1	3	2	11	1
	21	1.00E-08							
989	T34	Yayla305	Incompatible	Coleoptile	2	3	2	11	1
	21	1.00E-08							
993	T34	Yayla305	Incompatible	Coleoptile	4	3	2	11	1
	20	1.00E-08							
997	T34	Yayla305	Incompatible	Coleoptile	5	3	2	11	1
	21	1.00E-08							
999	T34	Yayla305	Incompatible	Coleoptile	6	3	2	11	1
	20	1.00E-08							
1001	T34	Yayla305	Incompatible	Coleoptile	7	3	2	11	1
	21	1.00E-08							

1123	T34	Yayla305	Incompatible	Crown	1	3	3	12	1	42
	0.008779592									
1124	T34	Yayla305	Incompatible	FirstLeaf	1	3	3	12	1	
	42	1.00E-08								
1125	T34	Yayla305	Incompatible	SecondLeaf	1	3	3	12	1	
	42	1.00E-08								
1127	T34	Yayla305	Incompatible	Crown	2	3	3	12	1	45
	0.00498452									
1128	T34	Yayla305	Incompatible	FirstLeaf	2	3	3	12	1	
	45	0.055645732								
1129	T34	Yayla305	Incompatible	SecondLeaf	2	3	3	12	1	
	45	0.002907521								
1131	T34	Yayla305	Incompatible	Crown	3	3	3	12	1	38
	1.00E-08									
1132	T34	Yayla305	Incompatible	FirstLeaf	3	3	3	12	1	
	38	1.00E-08								
1133	T34	Yayla305	Incompatible	SecondLeaf	3	3	3	12	1	
	38	0.008239087								
1135	T34	Yayla305	Incompatible	Crown	4	3	3	12	1	39
	1.00E-08									
1136	T34	Yayla305	Incompatible	FirstLeaf	4	3	3	12	1	
	39	1.00E-08								
1137	T34	Yayla305	Incompatible	SecondLeaf	4	3	3	12	1	
	39	1.00E-08								
1139	T34	Yayla305	Incompatible	Crown	5	3	3	12	1	46
	1.00E-08									
1140	T34	Yayla305	Incompatible	FirstLeaf	5	3	3	12	1	
	46	1.00E-08								
1141	T34	Yayla305	Incompatible	SecondLeaf	5	3	3	12	1	
	46	1.00E-08								

1143	T34	Yayla305	Incompatible	Crown	6	3	3	12	1	42
	0.000673618									
1144	T34	Yayla305	Incompatible	FirstLeaf	6	3	3	12	1	
	42	1.00E-08								
1145	T34	Yayla305	Incompatible	SecondLeaf	6	3	3	12	1	
	42	1.00E-08								
1147	T34	Yayla305	Incompatible	Crown	7	3	3	12	1	45
	0.017700707									
1148	T34	Yayla305	Incompatible	FirstLeaf	7	3	3	12	1	
	45	1.00E-08								
1149	T34	Yayla305	Incompatible	SecondLeaf	7	3	3	12	1	
	45	1.00E-08								
1151	T34	Yayla305	Incompatible	Crown	8	3	3	12	1	46
	0.012112687									
1152	T34	Yayla305	Incompatible	FirstLeaf	8	3	3	12	1	
	46	1.00E-08								
1153	T34	Yayla305	Incompatible	SecondLeaf	8	3	3	12	1	
	46	1.00E-08								
1155	T34	Yayla305	Incompatible	Crown	9	3	3	12	1	41
	1.00E-08									
1156	T34	Yayla305	Incompatible	FirstLeaf	9	3	3	12	1	
	41	0.185814364								
1157	T34	Yayla305	Incompatible	SecondLeaf	9	3	3	12	1	
	41	1.00E-08								
1559	T34	Yayla305	Incompatible	Crown	2	3	5	32	7	
	125	1.00E-08								
1560	T34	Yayla305	Incompatible	FirstNode	2	3	5	32	7	
	125	1.00E-08								
1561	T34	Yayla305	Incompatible	FirstLeaf	2	3	5	32	7	
	125	1.00E-08								

1562	T34	Yayla305	Incompatible	SecondNode	2	3	5	32	7
	125	1.00E-08							
1563	T34	Yayla305	Incompatible	Devhead	2	3	5	32	7
	125	1.00E-08							
1564	T34	Yayla305	Incompatible	FirstNode	2	3	5	32	7
	125	1.00E-08							
1565	T34	Yayla305	Incompatible	FirstLeaf	2	3	5	32	7
	125	1.00E-08							
1566	T34	Yayla305	Incompatible	SecondNode	2	3	5	32	7
	125	1.00E-08							
1567	T34	Yayla305	Incompatible	Devhead	2	3	5	32	7
	125	1.00E-08							
1568	T34	Yayla305	Incompatible	FirstNode	2	3	5	32	7
	125	1.00E-08							
1569	T34	Yayla305	Incompatible	FirstLeaf	2	3	5	32	7
	125	1.00E-08							
1570	T34	Yayla305	Incompatible	SecondNode	2	3	5	32	7
	125	1.00E-08							
1571	T34	Yayla305	Incompatible	ThirdNode	2	3	5	32	7
	125	1.00E-08							
1572	T34	Yayla305	Incompatible	Devhead	2	3	5	32	7
	125	1.00E-08							
1467	T34	Yayla305	Incompatible	FirstNode	1	3	5	33	7
	123	1.00E-08							
1468	T34	Yayla305	Incompatible	SecondNode	1	3	5	33	7
	123	1.00E-08							
1469	T34	Yayla305	Incompatible	FirstLeaf	1	3	5	33	7
	123	1.00E-08							
1470	T34	Yayla305	Incompatible	ThirdNode	1	3	5	33	7
	123	1.00E-08							

```
1471  T34  Yayla305  Incompatible FourthNode  1  3  5  33  7
      123  1.00E-08
1472  T34  Yayla305  Incompatible Devhead     1  3  5  33  7
      123  1.00E-08
1473  T34  Yayla305  Incompatible FirstNode   1  3  5  33  7
      123  1.00E-08
1474  T34  Yayla305  Incompatible SecondNode  1  3  5  33  7
      123  1.00E-08
1475  T34  Yayla305  Incompatible FirstLeaf   1  3  5  33  7
      123  1.00E-08
1476  T34  Yayla305  Incompatible ThirdNode   1  3  5  33  7
      123  1.00E-08
1477  T34  Yayla305  Incompatible Devhead     1  3  5  33  7
      123  1.00E-08
1478  T34  Yayla305  Incompatible FirstNode   1  3  5  33  7
      123  1.00E-08
1479  T34  Yayla305  Incompatible FirstLeaf   1  3  5  33  7
      123  0.004771742
1480  T34  Yayla305  Incompatible SecondNode  1  3  5  33  7
      123  1.00E-08
1481  T34  Yayla305  Incompatible ThirdNode   1  3  5  33  7
      123  1.00E-08
1482  T34  Yayla305  Incompatible FourthNode  1  3  5  33  7
      123  1.00E-08
1483  T34  Yayla305  Incompatible Devhead     1  3  5  33  7
      123  1.00E-08
;
run;

*Create GS categories **/ ;
```

```
proc format;
    value gsf   1 = '1'
                2 = '2'
                3 = '3'
                5 = '4';
run;

*Sort the data frame **/ ;

proc sort data=cov;
by trial treatment cultivar gs;
run;

*Create means table **/ ;

proc means data=cov;
var fdna;
output out=really_mean mean=;
by trial treatment cultivar gs;
run;

*Classic glimmix model – no covariates **/ ;

proc glimmix plots=(studentpanel) data=really_mean;
    format gs gsf.;
    class cultivar treatment trial gs;
    model fdna = cultivar treatment cultivar*treatment
            gs treatment*GS cultivar*GS treatment*cultivar*GS /dist=lognormal;
    random trial*cultivar*treatment*gs;
    *random zadoks/type=cs;
    lsmeans treatment*gs/plots=mean(cl join sliceby=gs) pdiff slicediff=gs;
```

```
        lsmeans cultivar*treatment*gs/plots=mean(cl join sliceby=gs) pdiff slicediff=gs;

run;
```

For the Spearman Correlation Between Tissues and Disease Outcome

```
*Input data **/ ;

data leafone;
input trial race$ cult$ gs leafn fdna bkern;
cards;
1    L18    HeinesVII    3    5    0.10    0.50
1    L18    Yayla305     3    4    0.14    1.00
1    T34    HeinesVII    3    3    0.06    0.25
1    T34    Yayla305     3    4    0.03    0.00
2    L18    HeinesVII    3    6    0.07    0.08
2    L18    Yayla305     3    2    0.16    0.93
2    T34    HeinesVII    3    8    0.02    0.42
2    T34    Yayla305     3    7    0.04    0.00
3    L18    HeinesVII    3    8    0.08    0.08
3    L18    Yayla305     3    8    0.08    0.35
3    T34    HeinesVII    3    9    0.10    0.01
3    T34    Yayla305     3    9    0.03    0.00
;
run;

data leaftwo;
input trial race$ cult$ gs leafn fdna bkern;
cards;
2    L18    HeinesVII    5    3    0.00    0.08
2    L18    Yayla305     5    3    0.28    0.93
```

```
2	T34	HeinesVII	5	2	0.00	0.42
2	T34	Yayla305	5	1	0.00	0.00
3	L18	HeinesVII	5	6	0.00	0.08
3	L18	Yayla305	5	7	0.17	0.35
3	T34	HeinesVII	5	6	0.00	0.01
3	T34	Yayla305	5	6	0.00	0.00
;
run ;

data deadhead;
input trial treatment$ cultivar$ fdna di;
cards;
2	L18	HeinesVII	0.01	0.08
2	L18	Yayla305	0.48	0.93
2	T34	HeinesVII	0.00	0.42
2	T34	Yayla305	0.00	0.00
3	L18	HeinesVII	0.01	0.08
3	L18	Yayla305	0.15	0.35
3	T34	HeinesVII	0.00	0.01
3	T34	Yayla305	0.00	0.00
;
run;

*Run Spearman correlations **/ ;

proc corr spearman data=leafone;
var fdna bkern;
run;

proc corr spearman data=leaftwo;
var fdna bkern;
```

```
run;

proc corr spearman data=deadhead;
var fdna di;
run;
```