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Master Thesis

Breeding for organic agriculture: Phenotypic evaluation and DNA marker screening for common bunt resistance genes *Bt9* and *Bt11* in winter wheat populations

Submitted by

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Declaration under oath

Herewith I affirm that I produced this Master thesis myself. No resources other than those cited were used. Phrasings and thoughts which have been adopted directly or indirectly from foreign resources are marked. This thesis has never been submitted anywhere before.

Abstract

Common bunt (caused by the fungi *Tilletia caries* and *T. laevis*) has been a problem in agricultural wheat production for over 2000 years, periodically leading to devastating losses in quality and yield as well as to outbreaks of famine. With the development and widespread application of chemical seed treatments this wheat disease could be controlled successfully, eventually leading to its near disappearance in some regions. However, common bunt re-emerged in organic agriculture over the past 30 years, as the application of fungicidal seed treatments is banned in organic production systems. To control common bunt in organic wheat production, plant breeders have been focusing on host resistance by identifying resistance genes and incorporating them into lines, cultivars and populations. During this master thesis, 494 $F_{5:7}$ recombinant inbred lines (RILs) divided into two mapping populations were screened for the resistance genes *Bt9* and *Bt11* using phenotypic and genotypic approaches. The mapping populations resulted from crosses between resistant parental lines PI166910 and 702-1102C and susceptible cultivars 'Rainer' and 'Lukullus'.

In the course of field scoring, date of heading, plant height, presence or absence of awns, brown rust incidence as well as common bunt incidence were assessed. Analysis of these data revealed that resistance towards common bunt was relatively high with 72.68 % of all tested lines of mapping population PI166910*SP and 40.22 % of mapping population 702-1102C*SP showing zero to 4.99 % common bunt incidence. Further statistical examination demonstrated that lines expressing high common bunt resistance levels as well as other favorable traits can be selected in both mapping populations.

Genotypically, lines were analyzed with KASP and SSR markers in order to screen for common bunt resistance genes *Bt9* and *Bt11*. Testing for polymorphism of markers revealed two suitable KASP markers for *Bt9* on chromosome 6D: RAC875_c48570_361 and BobWhite_c13435_700. However, in the course of this thesis no polymorphic DNA markers indicative of *Bt11* were available and therefore, screening for resistance gene *Bt11* was not possible. Analysis of common bunt resistance within each mapping population using KASP markers revealed a higher precision with marker BobWhite_c13435_700 (471.008508 Mbp on chromosome 6D) leading to the assumption that an analysis with this marker appears promising to perform marker assisted selection for the presence or absence of common bunt resistance gene *Bt9*.

Keywords: common bunt, *T. caries*, *T. laevis*, organic wheat breeding, marker analysis, KASP, resistance genes, *Bt9*, *Bt11*

Zusammenfassung

Weizensteinbrand (verursacht durch *Tilletia caries* und *T. laevis*) stellt seit über 2000 Jahren ein Problem in der Weizenproduktion dar, indem er periodisch zu verheerenden Verlusten hinsichtlich Qualität und Ertrag sowie zum Ausbruch von Hungersnöten führte. Mit der Entwicklung und dem großflächigen Einsatz von Saatgutbeizungen konnte die Pilzkrankheit erfolgreich unter Kontrolle gebracht werden. Innerhalb der letzten 30 Jahre ist Weizensteinbrand jedoch in der biologischen Landwirtschaft erneut aufgetreten, da Saatgutbeizungen auf ein Minimum reduziert wurden. Um Steinbrand in der biologischen Weizenproduktion zu bekämpfen, haben Pflanzenzüchter ihren Fokus auf Wirtsresistenz gelegt, indem sie Resistenzgene identifizieren und diese in Sorten einkreuzen. Im Zuge dieser Masterarbeit wurden 494 $F_{5:7}$ rekombinante Inzuchtlinien, die in zwei Kartierungspopulationen unterteilt sind, mit phänotypischen und genotypischen Methoden auf Steinbrandresistenz getestet. Die Kartierungspopulationen gingen aus Kreuzungen zwischen den resistenten Elternlinien PI166910 und 702-1102C und den anfälligen Sorten „Rainer“ und „Lukullus“ hervor.

Phänotypisch wurden das Datum des Ährenschiebens, die Wuchshöhe, die Begrannung, der Braunrostbefall und Steinbrandbefall bonitiert. Die Analyse dieser Daten ergab eine hohe Steinbrandresistenz in beiden Kartierungspopulationen. Weitere statistische Auswertungen zeigten, dass in jeder der beiden Populationen Linien mit hoher Steinbrandresistenz gleichzeitig mit anderen gewünschten Eigenschaften selektiert werden können.

Um die Linien auf das Vorhandensein der Resistenzgene *Bt9* und *Bt11* zu testen, wurden die Linien mit DNA-Markern überprüft. Unter mehreren getesteten KASP-Markern auf Chromosom 6D erwiesen sich zwei als polymorph: RAC875_c48570_361 und BobWhite_c13435_700. Jedoch konnten im Zuge dieser Masterarbeit keine polymorphen Marker für die Testung auf das Resistenzgen *Bt11*, das möglicherweise auf Chromosom 3B liegt, identifiziert werden, wodurch die Präsenz von *Bt11* in den Populationen nicht geprüft werden konnte. Die Analysen beider Populationen mit KASP-Markern ergaben eine etwas höhere Präzision hinsichtlich Steinbrandresistenz mit Marker BobWhite_c13435_700. Dies führt zu der Annahme, dass dieser Marker für eine markergestützte Selektion auf Anwesenheit von Resistenzgen *Bt9* aussichtsreich erscheint.

Stichworte: Weizensteinbrand, *T. caries*, *T. laevis*, biologische Weizenzüchtung, Marker-Analyse, KASP, Resistenzgen, *Bt9*, *Bt11*

Contents

Glossary

ANOVA	Analysis of variance
BBCH	A system to code the phenological growth stages of plants
BLUEs	Best linear unbiased estimates
BOKU	University of Natural Resources and Life Sciences
BRI	Brown rust incidence [%]
CBI	Common bunt incidence [%]
DH	Double haploid
dNTP	Deoxyribonucleotide triphosphate
DOH	Date of heading [days after May 1st]
DNA	Deoxyribonucleic acid
FAM	Fluorescein amidite
FRET	Fluorescence resonant energy transfer
InDel	Insertion and deletion
KASP	Kompetitive allele specific PCR
MAS	Marker assisted selection
n	Number of individuals
p	p-value
PCR	Polymerase chain reaction
PH	Plant height
QTL	Quantitative trait locus
RIL	Recombinant inbred line
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats
SP	Susceptible parent
α	Type I error

1 Introduction

1.1 Common bunt – an everlasting threat

Common bunt is one of the most destructive wheat diseases worldwide (Goates, 1996). The bunt and smut fungi probably originated from the Fertile Crescent, together with wheat and wheat relatives (Saari and Mamluk, 1996). This theory is supported by the many resistance genes collected in this region from wild species of wheat and grasses (Gaudet and Menzies, 2012). From this area, common bunt spread all over the world, mostly by seeds. Wind dispersal was probably not important in early times, as harvesting was still done by hand. In modern days, distribution through wind is more relevant, as harvesters release spores into the atmosphere, allowing them to be carried over long distances (Saari and Mamluk, 1996). The first reports on smut of wheat date back to the third century before Christ (Spieß et al, 2015), other sources indicate the presence of common bunt since ancient wheat production (Saari et al, 1996). Today, the disease occurs all over the world mainly on winter-sown wheat with optimal infection temperatures between 6 and 7 °C (Brandstetter and Weinhappel, 2011; Goates, 1996; Spieß et al., 2015). Only with the introduction of hot water treatments and seed-washing, a first method providing control against common bunt became available. Seed treatments with organomercury were applied since the 1930s to curb common bunt incidences (Spieß, 2015; Clark and Cockerill, 2011). However, in the first half of the 20th century common bunt persisted as a constant obstacle in agricultural production, especially in the USA (Goates, 1996). Almost complete control of common bunt was achieved using chemical seed treatment with fungicides like hexachlorobenzenes, which act effectively against seed- and soil-borne inoculum. Due to the implementation of these fungicides, common bunt incidence was decreasing to a minimum by the 1950s (Goates, 1996; Hoffmann, 1982). Changes in agricultural production during the past decades have led to a more organic and sustainable cultivation, resulting in a reduced application of chemical seed treatments. This facilitated a re-emergence of common bunt in low-input and organic wheat over the last 30 years, especially in northern and western Europe (Matanguihan et al., 2011). Contaminated farm-saved seed seems to be the main cause of this anew spreading of common bunt (Weinhappel and Riepl, 2013).

1.2 Common bunt – pathogens and their biology

Common bunt is a fungal disease caused by *Tilletia caries* (D.C.) Tul. & C. Tul. (syn. *Tilletia tritici* (Bjerk.)) G. Winter. and *Tilletia leavis* J. G. Kühn (syn. *T. foetida* (Wallr.) Liro) (Matanguihan et al., 2011) which periodically led to extremely high losses in yield and quality of wheat (Goates, 1996; Fischer and Holton, 1957). Both fungi are obligate parasites. They

are heterobasidiomycetes in the order Ustilaginales, which belong to the family of the Tilletiaceae (Goates, 1996). Their primary host is wheat (*Triticum aestivum* L.), especially when planted in autumn due to the favorable environmental conditions for infection (Matanguihan et al., 2011). Nevertheless, common bunt can also occur in spring wheat. More than 70% of the spikes can be infected when plants are grown in environments with high inoculum levels and no precautions like host resistance or chemical seed treatment are taken. Typically, disease incidence is equal to yield loss, as healthy kernels are replaced with bunt balls called sori, which contain spores. *T. caries* and *T. laevis* are nearly identical regarding morphology, germination requirements, symptoms and life cycles. However, *T. caries* usually occurs in the Northwest of Europe, whereas *T. laevis* is more common in Eastern Europe (Gaudet and Menzies, 2012; Goates, 1996; Matanguihan et al., 2011). Although both fungi show similar morphology, their teliospores differ substantially in cell wall characteristics. Teliospores of *T. caries* show reticulate exospores with polygonal structures of 0.5 to 1.5 µm depth which form a net-like pattern, while those of *T. laevis* have smooth exospores. Additionally, teliospores of *T. caries* are 14 to 23.5 µm in diameter, have thin walls (0.5 - 1.5 µm) and a brittle pericarp. They are primarily globose or ovoid and show a light pale yellow to grey or reddish-brown color (Figure 1). *T. laevis* forms teliospores with a diameter of 14 to 22 µm (sometimes only 13 µm) and thin walls (0.5 - 1.4 µm). They are globose, ovoid and occasionally elongate and appear light pale to dark brown (Figure 1) (Durán and Fischer, 1961; Goates, 1996).

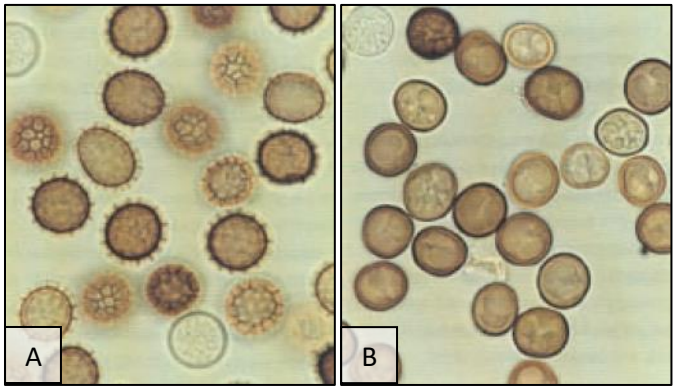

Figure 1. *Teliospores of T. caries (A); teliospores of T. laevis (B) (Goates, 1996)*

T. caries and *T. laevis* may hybridize with each other as well as with the causal agent of dwarf bunt, *T. controversa* (Goates, 1996). Dwarf bunt is a disease solely caused by soil-borne teliospores. Germination is favored by weeks of cool temperatures provided by a continuous snow cover. Beneath this snow cover, teliospores germinate and colonize already emerged seedlings. Resistance to both common bunt and dwarf bunt in wheat is partly controlled by the same set of genes, however, certain resistance loci are only effective against one of the

diseases (Goates, 2012; Muellner et al., 2020). Common bunt and dwarf bunt may appear in the same field, but rarely on the same wheat plant. Nevertheless, pathogens of both diseases have been observed in the same sorus (Fischer and Holton, 1957).

Infection with common bunt usually occurs via seed-borne teliospores which colonize the seeds shortly after they germinate (Figure 1) (Goates, 1996; Goates, 2012). Teliospores of *T. caries* and *T. laevis* germinate in the soil or on the seed and form a promycelium; long promycelia are stimulated by moisture (Goates, 2012; Saari and Mamluk, 1996). From the tip of the promycelium, primary sporidia emerge to form a tight bundle. Primary sporidia have two mating types, labelled "+" and "-", respectively. Two primary sporidia of opposite mating types fuse and become so-called H-bodies (Figure 2). H-bodies produce secondary sporidia and infection hyphae (Figure 2), which form an appressorium (Goates, 1996; Goates, 2012; Gaudet and Menzies, 2012). Wheat seedlings are invaded by infection hyphae before they emerge from the soil. Infection hyphae penetrate the coleoptile of both resistant and susceptible cultivars. For the disease to develop, hyphae must reach the apical meristem before internode elongation; i.e., in resistant plants infection hyphae do not stretch to the apical meristem (Goates, 1996; Saari and Mamluk, 1996). The mycelium of the fungus infects the growing point of wheat seedlings and consequently systematically infects the wheat plant until eventually young spikelets are permeated (Mathre, 2000; Saari and Mamluk, 1996). Developing wheat kernels are contaminated with mycelium developing teliospores, leading to kernels being transformed into bunt balls (sori). During harvesting, mature sori break and release teliospores which disseminate and contaminate healthy seeds as well as the soil (Saari and Mamluk, 1996). Teliospores adhering to seeds are viable for up to 20 years when stored in a dry place (Spieß et al., 2015). Common bunt infection can also occur via soil-borne teliospore inoculum which has become more prominent. A decrease in deep ploughing due to conserving techniques allowed soil-borne teliospores to remain germinable for several years (Goates, 1996; Spieß et al., 2015).

Common bunt incidence is favored by soil moistures ranging from near the permanent wilting point to field capacity. Optimal moisture is reached midway among those extremes, at 25 to 30 % (Fischer and Holton, 1957; Goates, 1996). Soil temperatures below the optimum of wheat germination are favorable to common bunt infections because seeds germinate more slowly and therefore take longer to emerge from the soil, giving teliospores more time to infect the plant (Fischer and Holton, 1957). The general optimum for infection with *T. caries* and *T. laevis* is between 5 and 10 °C soil temperature (Fischer and Holton, 1957; Goates, 1996). Infections with seed-borne inoculum occur at lower soil temperatures than with soil-borne teliospores (Goates, 1996). Generally, early fall seedings with still relatively warm soil temperatures will

result in less bunt incidence. Nevertheless, these environmental conditions facilitate the occurrence of viruses like cereal yellow dwarf virus (AGES, 2021; Fischer and Holton, 1957). Unfavorable for the fungus are very sandy soils as well as clay or high soil acidity. Typically, an optimal soil for infection is a mineral soil with a clay base, high humus content and a neutral pH (Fischer and Holton, 1957).

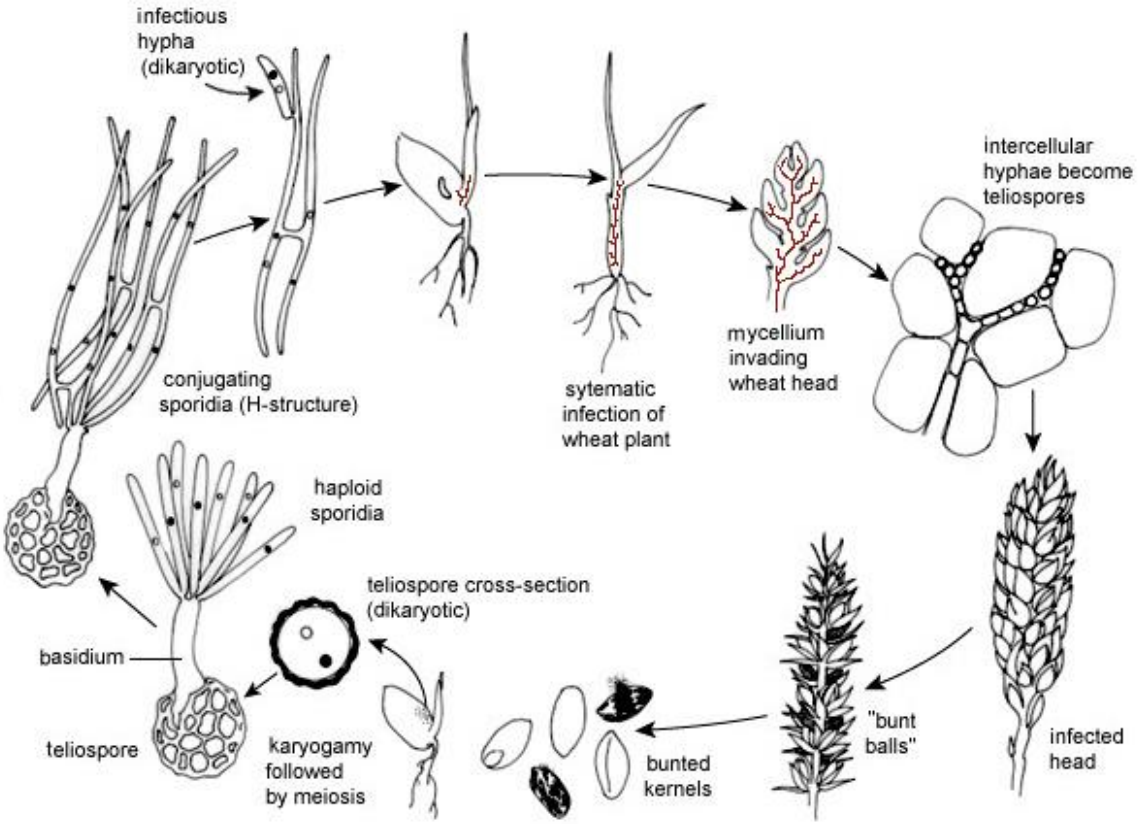

Figure 1. *Disease cycle of common bunt (T. caries and T. laevis) (Mathre, 2000)*

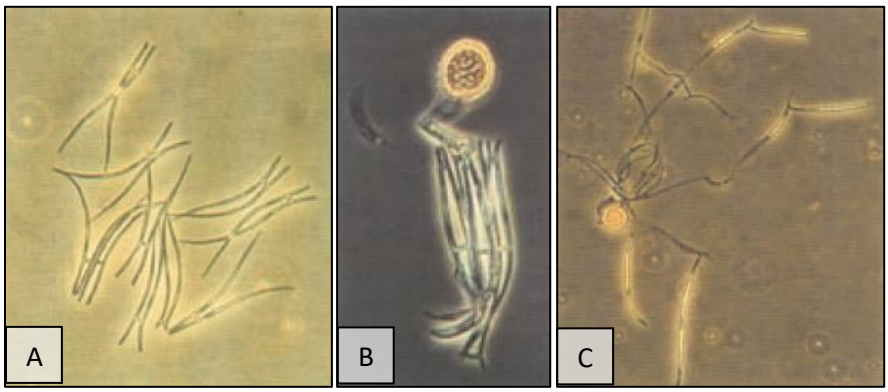

Figure 2. *H-bodies of primary sporidia of T. caries (A); germinated teliospore of T. caries with promycelium, primary sporidia, and H-bodies (B); secondary sporidia of T. caries (C) (Goates, 1996)*

Symptoms of common bunt frequently only appear after heading with the beginning of sporulation. Immature infected plants show spikes with a darker green or bluish-green color compared to healthy spikes. In a mature stage, spikes may have a slightly lighter color in contrast to healthy ones. Diseased plants are usually up to one third shorter and their anthers may be stunted, inhibiting them to extrude from the spelts (Bradstetter and Weinhappel, 2015; Goates, 1996; Spieß et al., 2015). Florets may be flared, pollen is nonviable and young ovaries show green walls (Goates, 1996). Healthy wheat kernels are replaced by bunt balls (sori), partial infestation of spikes is not uncommon (Brandstetter and Weinhappel, 2011; Spieß et al., 2015). Immature sori are still soft and contain a black, greasy mass emitting a fishy odor, giving common bunt its typical name “stinking smut”. The fishy odor originates from the formation of trimethylamine and ammonium compounds and may already be noticed with contamination levels of only 0.1 %. With progressing maturity, sori become harder and the greasy mass turns into a powder of teliospores. The smell may vanish and can therefore no longer be considered a safe characteristic to identify common bunt infections when plants become mature (Goates, 1996; Matanguihan et al., 2011; Spieß et al., 2015).
In contrast, plants infected with dwarf bunt are stunted (up to two thirds shorter), sori are harder and spreading of awns is severe (Brandstetter and Weinhappel, 2011).

1.3 Control of common bunt

In this chapter, the historical background and countermeasures against common bunt are described.

1.3.1 History of bunt control

In 1750, Mathieu Tillet performed experiments involving wheat seeds, which he coated with a black powder and observed that those seeds formed plants with smutted heads. He also planted wheat seeds without this black dust, which developed no or little smutted heads (Fischer and Holton, 1957; Matanguihan et al., 2011). Thereby he demonstrated that the smut spores were infective. Tillet discovered the cause of the disease along with a way to prevent it. For that, he washed wheat seeds in water, lye solutions, cattle urine, copper sulfate and lime and salt, all of which helped to suppress the disease. With this research, Tillet built the foundation for a new science – plant pathology – and was honored by smut of wheat being named *Tilletia* (Matanguihan et al., 2011). From the 1900s to the 1960s, management of common bunt was studied intensively, including pathogen genetics, pathogenic races, the effect of seeding dates, survival of spores in the soil and seed treatments (Bruehl, 1989). Wheat cultivars resistant to common bunt were first developed by W. J. Farrer by applying systematic breeding methods (Fischer et al., 1957). Subsequently, a search for more resistant

cultivars was conducted to start a breeding program for common bunt (Tisdale et al., 1925). To achieve greater knowledge about bunt resistance, Gaines screened the germplasm of wheat (Gaines, 1920), studied the genetics of common bunt resistance (Gaines, 1923), and discovered physiologic races of common bunt (Gaines, 1928a; Gaines, 1928b). Eventually, by the middle of the 20th century the disease almost disappeared due to resistant cultivars and effective chemical seed treatments (Menzies et al., 2009) i.e., fungicides like hexachlorobenzenes (Holton et al., 1954).

1.3.2 Chemical seed treatments

The widespread use of chemical seed treatments represented the most abundant measure to successfully contain seed- and soil-borne common bunt. Composition and substances changed over time from formaldehyde, copper carbonate, organic mercuries and polychlorobenzenes to finally systemic fungicides such as carboxin (Hoffmann, 1982; Mathre et al., 2001). Until the development of polychlorobenzenes, soil-borne inoculum could not be controlled with chemical seed treatments. Hexachlorobenzenes, however, allowed the effective management of both seed-borne and soil-borne teliospores, leading to a significant diminishment of common bunt (Hoffmann, 1982; Hoffmann and Waldher, 1981). Chemical seed treatments act in different ways in the wheat plant. Some, for example, act as sterol biosynthesis inhibitors, others inhibit RNA synthesis by disrupting the incorporation of uridine into RNA, or they stop nuclear division by interfering with microtubule assembly by binding to the protein tubulin (Mathre et al., 2001). Homogenous seed treatment distribution on the seeds is of utmost importance, so that no parts of the grains remain untreated and therefore vulnerable to infection (Voit et al., 2012). In Austria, chemical seed treatments contain the following active components: Difenoconazol, Fludioxonil, and Sedaxan (Bundesamt für Ernährungssicherheit, 2021). Currently, all available chemical seed treatments are effective against common bunt. Their use can be reduced or omitted when seeds are not contaminated. Levels of seed contamination can be tested in special laboratories offering this analytical service (Brandstetter and Weinhappel, 2011; Clark and Cockerill, 2011; Voit et al., 2012). Some strains of common bunt may develop a certain level of resistance towards individual chemical seed treatment fungicides. Therefore, a continuous production of newly designed seed treatments is necessary (Hoffmann, 1982; Hoffmann and Waldher, 1981).

1.3.3 Organic and physical seed treatments and biological control

Over the past decades, common bunt was efficiently controlled by chemical seed treatments, however, they do not comply with the regulations in place for organic agriculture. Therefore,

research on alternative countermeasures and prophylaxes like physical and organic seed treatments as well as biological control strategies was and is being conducted (Klaedtke et al., 2021; Matanguihan, 2011).

Physical seed treatment methods such as hot water treatments are used to control a range of seed-borne pathogens of wheat and barley including the bunt fungi. Contaminated seeds are soaked in hot water and subsequently re-dried without negatively influencing germination of the seeds. However, due to high costs and efforts accompanying this method, it is only rarely used (Borgen, 2004; Koch et al., 2006; Matanguihan et al., 2011). Borgen (2005) cleansed contaminated seeds with an air-screen cleaner and subsequently brushed the seeds, reducing the number of spores by 99.5 %. The longer infected seeds were treated, the higher was the effect of the method (Borgen, 2005). Centrifuging seeds at high speed with a Sigma cleaner also showed promising results (Borgen, 2004). At the Swedish University of Agricultural Sciences, a treatment with hot, humid air was developed to destroy seed-borne fungi such as *T. caries* (Matanguihan et al, 2011).

Tillecur®, a yellow mustard flour, is an organic plant strengthening agent, which is applied as a slurry to contaminated wheat seeds before planting (Borgen, 2004; Matanguihan et al., 2011; Waldow and Jahn, 2003). Koch et al. (2006) compared the efficacy of this treatment to commercial and noncommercial biological control agents, none of which were as powerful as Tillecur®. Compared to seed treatments with hot water, Tillecur® was more effective in bunt control by reducing disease incidence, although it did not always suppress common bunt development (Waldow and Jahn, 2003).

Powdered skimmed milk was used by El-Naimi et al. (2000) as an organic seed treatment. Experiments over four years showed 96 % less common bunt incidence when seeds were treated with skimmed milk powder prior to bunt inoculation. Moreover, this alternative treatment was as effective as the chemical seed treatment in this experiment (Vitavax-200). This may be due to an increased antagonistic potential of microorganisms in the soil, or because teliospore germination is inhibited through the production of toxic metabolites (El-Naimi et al., 2000). However, skimmed milk powder may slow down emergence of the seedlings (Winter et al., 2001).

Borgen and Nielsen (2001) used 5 % acetic acid to treat previously contaminated seeds. Over two years, common bunt incidence could be reduced by 96 and 92 %, respectively. However, with increasing amounts of acetic acid applied on the seeds, germination speed was significantly decreasing. Sholberg et al. (2006) fumigated already inoculated wheat seeds with acetic acid vapor for one hour at 20 °C. Common bunt incidence was successfully reduced by

42 and 72 %, respectively, with 2 g/kg applied acetic acid. However, the higher the concentration and duration of the treatment, the less tillers were formed.

Biological control of *T. caries* can be achieved with the bacterial strain *Pseudomonas chlororaphis* MA 342. Seeds naturally infested with common bunt were treated with this biopesticide. Over four years of treatment only a mean of 4.3 infected spikes per m² were observed. *Pseudomonas chlororaphis* was later developed into Cedomon® and Cerall®, two commercial biopesticides (Johnsson et al., 1998; Matanguihan et al., 2011).
Dromph and Borgen (2001) tested the viability of common bunt teliospores after their ingestion by collembolans, which were common in the experimental region. They observed that after ingestion and excretion of teliospores, their germination rates decreased from 77.2 % to below 3 % compared to the control, reducing wheat infection levels drastically.
Biological control in general seems to be a controversial topic: on the one hand plant juices can be used to control certain diseases, on the other hand it is prohibited to use chemical compounds extracted from these plants and apply them in a higher dosage than they would naturally occur. Similarly, it is not recommended to introduce microorganisms into the soil where they are not usually found, as they may disturb the already existing soil flora (Borgen and Davanlou, 2000). Biological control measures are effective, however, they do not provide sufficient protection when only one of them is applied. A combination of different control strategies, for example mustard seed powder combined with brushing of the seeds, is necessary to avoid disease incidences. Nevertheless, this is time consuming and accompanied by higher costs (Wiik, 2021). Generally, organic seed treatments are an alternative to chemical seed treatments, however, in years with higher contamination levels, the efficiency of organic treatments is only about 65 % compared to chemical ones (Voit et al., 2012).

1.3.4 Cultural practices

Common bunt is a seed-borne disease, which means the spores remain on the seeds after harvest and the fungus develops when the crop is planted again (Goates, 2012). Therefore, only healthy seeds of the best quality (“Z-Saatgut” in Austria) should be sowed. If farmers intend to use their own seed it is recommended to test it before sowing, this service is offered by special laboratories (Brandstetter and Weinhappel, 2011; Voit et al., 2012). Healthy seed leads to accelerated germination and improved durability (Spieß et al., 2015). Hosts of common bunt can be amongst others various grasses, therefore, boundary ridges pose risks and should be mowed prior to the crop reaching maturity (Goates, 1996; Spieß et al, 2015). To avoid contamination via the soil, crop-rotation of two years between winter wheat or winter

spelt must be maintained (Brandstetter and Weinhappel, 2011). Adhering to optimal regional sowing times and shallow sowing depths as well as maintaining a beneficial condition of the soil are crucial to secure rapid germination and growth of the plant to minimize the risk of infection. Intensive organic fertilizing, for example with manure, increases soil activity and has phytosanitary effects (Spieß et al, 2015). Contamination with common bunt happens primarily due to custom sowing and harvesting, when one machine is operated on many different fields with different disease levels without decontaminating the machine between individual sites. Cleaning of seeders and harvesters but also storage facilities and conveyors is of utmost importance to maintain a healthy crop. During harvest, sori can break, spores can scatter and thereby pollute other grains, straw, soil, machines and surrounding fields (Brandstetter and Weihnappel, 2011). It has also been observed that after harvesting, the teliospore contamination level in the soil was increasing. This represents a latent risk of infection for wheat production (Voit et al., 2012). Due to contaminated harvested seeds (with a fishy smell), farmers cannot sell their cereals for the full price but are faced with a markdown. Sometimes the seeds are washed with hot water by the employees of the warehouse, however, this is expensive and therefore only done with premium-quality organic grains (Brandstetter and Weinhappel, 2011). In case the crop is contaminated and not cleaned, it can no longer be used and processed for human or animal consumption, moreover, it must be fermented in biogas plants or destroyed by fire in suitable facilities (Brandstetter and Weinhappel, 2011).

1.3.5 Host resistance and plant breeding

Until the 1950s, Pacific Northwestern breeding programs of the United States were focused on host resistance. By that time, soil-borne inoculum could not be controlled with seed treatments and breeders had to rely on resistant cultivars. However, host resistance was overcome relatively fast by previously undetected races of the pathogens. Therefore, in the late 1950s a cultivar with two resistance genes was developed, which provided protection against all races known at that time (Hoffmann, 1982). The development of chemical seed treatments and their extensive use entailed that breeding programs for common bunt resistance became irrelevant, were no longer pursued or given low priority (Matanguihan et al., 2011). Organic farmers still often have to rely on cultivars bred in conventional programs, which usually do not focus on host resistance against pathogens like the common bunt fungi. Therefore, in today's breeding programs basic research is done by monitoring common bunt incidence and pathogen races, screening cultivars for resistance, finding new sources of resistance and studying their mode of inheritance (Matanguihan et al., 2011).

Monitoring and identifying races of *T. caries* and *T. laevis* by observing virulence patterns of cultivars is crucial due to the gene-for-gene interaction between avirulence genes of the fungus and bunt resistance genes in wheat. Currently, there are 17 known common bunt resistance

genes, *Bt1* to *Bt15*, *BtP* and *BtZ*. New virulence patterns and races of common bunt are determined by inoculating and screening a differential set of wheat cultivars which monogenically harbour different known resistance genes. Infection with a certain common bunt isolate will then give a virulence pattern which is analyzed and compared. If a pattern does not match any of the known ones, a new race could be assumed. This information is vital for breeders, allowing them to incorporate the relevant resistance genes needed in a specific area into their breeding lines (Borgen et al., 2019; Gaudet and Puchalski, 1989; Goates, 2012; Hoffmann and Metzger, 1976; Matanguihan et al., 2011). Fofana et al. (2008) were the first to map QTL for common bunt resistance. They tested an F_1-derived DH population from the cross RL4452*AC Domain and obtained the location of three QTL: two of them were mapped to chromosome 1B and one to the long arm of chromosome 7A. To locate bunt resistance genes, knowledge about the genetic control of bunt resistance and the mode of inheritance is important (Goates, 2012).

The genetic base for resistance towards *T. caries* and *T. laevis* in wheat is limited. Most modern cultivars are susceptible to at least one of the known common bunt races (Martynov et al., 2004). Hoffmann (1982) pointed out the urgency of finding new sources of resistance which can provide a greater genetic diversity for breeding programs. Bonman et al. (2006) studied the geographic distribution of common bunt resistance. The area ranging from Serbia and Montenegro to Macedonia, Turkey and Iran seems to be the center for bunt resistance. Landraces collected in this region may be good sources of new resistance genes or may provide new gene combinations for bunt resistance. Other sources of bunt resistance could be found in related wheat species and genera as well as wild relatives (He and Hughes, 2003; Matanguihan et al., 2011). For example, Dumalasová and Bartoš (2010) tested several cultivars of species related to wheat: durum wheat, emmer, spelt and triticale. Generally, bunt infections were lower compared to the susceptible check. Durum wheat showed up to 6.2 % infected ears, emmer 5.6 % and one of the tested spelt wheat cultivars was also highly resistant. Triticale, however, was especially resistant with very low infection levels of only up to 2.0 %.

Screening studies have been conducted to obtain information on the levels of resistance in already registered cultivars. In Europe, most of the cultivars seem to be susceptible. In Serbia and Montenegro, only four out of 12 tested cultivars were resistant to the bunt fungi (Matanguihan et al., 2011; Rajkovic and Dolovac, 2006). Studies of Dumalasová and Bartoš (2006a, 2006b, 2007) showed a fluctuation of levels of resistance between cultivars, years and locations. Therefore, cultivars should be screened over a minimum of three years in different locations with a standardized source and dose of inoculum to obtain reliable results.

Gaudet and Puckalski (1989) observed that many of their tested cultivars (hard red spring wheats) showed satisfactory levels of resistance in the field but were susceptible in controlled

environments and they therefore discussed the occurrence of field resistance. As this type of resistance could not be expressed under controlled conditions, it is indicated that the tested cultivars do not harbor any of the *Bt* resistance genes. Instead, field resistance seems to be race non-specific and is weakened or lost in controlled environments. Involved resistance mechanisms are unknown, but probably occur due to disease escape.

As many resistant wheat cultivars unfortunately have agronomically poor traits, the main goal and challenge is to introgress common bunt resistance genes and simultaneously eliminate poor agronomic performance (Hajjar and Hodgkin, 2007). However, the development of new cultivars that are resistant to common bunt and adapted to organic agriculture will remain a time-consuming task, whether resistance is achieved through conventional breeding or with the support of molecular markers (Matanguihan et al., 2011).

1.4 Marker assisted selection

Phenotypic screening of individuals is time-consuming, as disease symptoms of common bunt are only visible at plant maturity. Therefore, molecular markers were introduced to assist in the process of screening for resistance to develop robust cultivars. Molecular markers can genotypically identify resistant and susceptible cultivars already at an early stage of plant growth, accelerating the screening process considerably. To develop cultivars with long-term resistance, pyramiding of bunt resistance genes with the assistance of markers could be beneficial (Bartoš et al., 2002; Matanguihan et al., 2011).

Molecular markers, such as KASP (kompetitive allele specific PCR) or SSR (simple sequence repeats, also called microsatellites) markers, are frequently used in plant breeding (Collard and Mackill, 2007). KASP markers are designed to have specific primers complementary to the targeted DNA sequence. With the use of KASP markers, single nucleotide polymorphisms (SNPs) and insertions and deletions (InDels) can be scored at specific loci (LGC, 2015). Microsatellites (or SSR markers) are short nucleotide sequences (one to ten base pairs), which are repeated frequently – typically five to 50 times – and distributed throughout the genome (Kumar, 2018; Kwok and Schmitt, 2002). They are very reliable, relatively cheap and highly polymorphic (Collard and Mackill, 2007). The number of repeats per locus can differ, hence, the sequences can vary in length. Therefore, these sequences are analyzed by determination of their sizes with gel electrophoresis and so, genotypes or alleles, respectively, can be distinguished (CD Genomics, 2021; Kwok and Schmitt, 2002).

Generally, essential advantages of marker assisted selection (MAS) over conventional phenotypic selection are:

- higher efficiency compared to phenotypic screening by saving time, effort and resources,

- selection of individuals is possible already at the seedling stage and
- selection of single plants (Collard and Mackill, 2007).

1.4.1 KASP genotyping assay

Genotyping assays of KASP markers consist of the following three components:

- **KASP assay mix** containing two allele-specific primers with unique tail sequences and one common reverse primer,
- **KASP master mix** containing two fluorescence resonant energy transfer (FRET) cassettes and
- **template DNA** with the target SNP (LGC, 2015a).

Primers in the KASP assay mix are simple unlabeled oligonucleotides. The 3' ends of both allele-specific primers are complementary to the target DNA sequence and have different regions at the 3' ends to detect the SNP. Tail sequences of these primers are not complementary to the template DNA; however, they are crucial for signal generation. For simplicity, allele-specific primers will be called "X" and "Y" while non-complementary sequences will be called "X tail" and "Y tail" in the following paragraphs. KASP master mix contains two fluorescently labeled reporter cassettes (FRET cassettes), each comprising a fluorophore (FAM or HEX) and a quencher preventing the production of undesired fluorescence signals. Each fluorophore is complementary to an X or Y tail, for example FAM would be complementary to the X tail and HEX to the Y tail. Additionally, KASP master mix also contains all other components typically needed for PCR (chemically inactivated Taq polymerase, deoxyribonucleotide triphosphates - dNTPs, salts and buffer) (LGC, 2015a).
For the KASP reaction, DNA sample, assay mix and master mix are loaded into the same well (see subsection 3.4.1. for exact process and volumes) and the KASP thermocycle protocol is run. In the first round, KASP Taq is activated by a hot start and template DNA is denatured. Allele-specific primers (X and Y) compete to bind to the template DNA. These primers need to match 100 % with the template DNA (except non-complementary tail sequences) to be able to bind. Common reverse primers also bind to the template DNA and KASP Taq extends all primers, copying only DNA containing SNPs. Non-complementary tail sequences (X tails and Y tails) will not be copied but incorporated into the PCR product. In the beginning of round two of the PCR, DNA (original template and PCR products of round one) is denatured and allele-specific and common reverse primers are annealed and get extended. X and Y tails incorporated in PCR products of round one are copied as well, resulting in so-called complements (complement to X tail will be called "X complement", complement to Y tail will be called "Y complement"). Round three again starts with denaturation of DNA (original template and PCR products of rounds one and two) and primers anneal and are extended. FAM and

HEX labeled oligonucleotides from the FRET cassettes will bind to X and Y complements and extend. The number of PCR products as well as of X and Y complements thereby increases exponentially with each round of PCR. KASP is an endpoint genotyping chemistry and can therefore only be read once the PCR reaction is finished. Depending on the alleles present in the DNA template, either FAM labeled oligonucleotides, HEX labeled oligonucleotides or both will be incorporated in the PCR products. For example, if template DNA is homozygous for allele A, primer X will bind and consequently X complements and FAM labeled oligonucleotides will be incorporated. If template DNA is heterozygous, both FAM and HEX signals will be generated. Before reading the fluorescence, samples must be cooled down below 40 °C. This leads to quenching of fluorescently labeled oligonucleotides which have not been incorporated into PCR products, so that these will not emit fluorescence interfering with the desired signal (LGC, 2015b). Relative fluorescence units measured on each well and processed by a BioRad software are displayed in a cartesian plot with FAM fluorescence on the x- and HEX fluorescence on the y-axis. This graphical display facilitates allelic discrimination as samples homozygous for allele 1 or 2, respectively, will form clusters along the two axes. Heterozygous samples emit both types of fluorescence and will be displayed along a diagonal through the center of the plot. To validate the results, non-template controls as well as controls for both homozygous as well as heterozygous allelic state need to be included for each marker (LGC, 2015c).

1.4.2 SSR genotyping assay

Microsatellites are tandem repetitive sequences, in which the repeating region contains one to ten nucleotides. The number of repeats is variable between populations, they can appear for instance as mono-, di-, tri- or tetranucleotides. Although microsatellites are unstable due to dividing cells, their length remains the same. Microsatellites are widely distributed across the genome and highly polymorphic – probably due to slippage replication. Additionally, they are locus-specific, codominant and PCR-based, which is why they do not require expensive laboratory equipment.

Specific primers are designed, microsatellites are amplified and polymorphisms are tested, i.e., genotyped. To design primers, a reference sequence of the target region is necessary. SSR motifs can be identified by using identification tools and locus-specific primers can be created. Once primers have been developed, their polymorphisms for the target gene must be validated using PCR amplification. Each SSR genotyping assay consists of the following components:

- DNA polymerase,
- reaction buffer,
- Magnesium ion ($MgCl_2$),
- dNTP mix (nucleotides),

- forward primer,
- reverse primer and
- genomic DNA.

36 cycles of PCR with specific temperature changes for denaturation, annealing and extension are performed. PCR products can be visualized with polyacrylamide or agarose gel electrophoresis as well as capillary electrophoresis (CD Genomics, 2019).

1.5 Breeding for organic agriculture

Organic breeding for new varieties is a holistic process. Therefore, breeding programs and cultivar development must comply with the principles of organic farming (Messmer et al., 2015). Organic agriculture as well as organic plant breeding and seed production rely on ecological and ethical values (Lammerts van Bueren et al., 2007; Wolfe et al., 2008). They concentrate on preserving the integrity of the plant, increasing genetic diversity, respect for crossing barriers and interactions of the plant with the living soil and climate. The main traits organic breeding programs concentrate on are resistance to soil- and seed-borne diseases, competitiveness against weeds, rapid youth development, good lodging resistance, nutrient use efficiency as well as quality-related features. These characteristics are rarely considered in conventional breeding programs resulting in an urgency for specially bred organic varieties with greater efficiency and yield stability. Genetically modified organisms are not allowed under organic conditions according to EC Regulation No 834/2007. This regulation also states that the mother plant of organic seeds must have been produced under organic conditions for at least one generation (Messmer et al., 2015; Lammerts van Bueren et al., 2011).

Nevertheless, more than 95 % of all cultivars used in low-input organic agriculture were bred under high-input conventional conditions. Although breeding goals and agronomic traits such as yield, baking quality and resistance to biotic and abiotic stress are the same in organic and conventional farming environments, those traits may not be expressed as intensively under low-input conditions when selection was done under high-input farming (Lammerts van Bueren et al., 2007). Furthermore, breeding for conventional agriculture focuses on traits which may lead to unfavorable side-effects in organic systems. Conventional breeding programs often introduce semi-dwarf genes resulting in short-straw cultivars. These lead to a higher need for inorganic nitrogen input and lower nutrient-use efficiency, a reduced root system and decreased competitiveness against weeds as well as reduced robustness against diseases when cultivated organically (Lammerts van Bueren et al., 2011). As the expression of the phenotype depends on the environment, cultivars used in organic agriculture should not be selected and bred under conventional conditions (Lammerts van Bueren et al., 2007).

Achieving a good yield stability can be complex in organic systems, as there is a great environmental variability over years and between locations (Wolfe et al., 2008). To obtain high yielding varieties with good yield stability, the focus lies on their adaptation to

- organic soil fertility management,
- organic weed management,
- organic pest and disease management,
- organic seed production management and to
- organic quality requirements (Lammerts van Bueren et al., 2007).

Due to the European Organic Seed Regulation (EC 1452/2003), the use of organic seeds in organic agriculture is becoming mandatory by stating that if there is enough organically produced seed available it should not be possible to use seeds not obtained by organic production methods (Fischler, 2003; Wolfe et al, 2008). Nevertheless, varieties for organic systems still may come from three different approaches. First, organic farmers may select their cultivars from conventional breeding programs, from which they choose the ones performing best under organic conditions. Further, they could obtain their seeds from breeding programs for organic agriculture. These breeding programs start with crosses specific for organic farming. The first selection steps, however, are performed under conventional conditions. Lastly, farmers may receive their varieties from breeding programs within organic agriculture. With this method, all breeding steps are executed under organic conditions (Wolfe et al., 2008). Löschenberger et al. (2014) conducted a ring test to identify direct and indirect selection strategies for organic agriculture. The experiment revealed that higher selection efficiency at lower cost may be accomplished by combining information obtained from organic, conventional low-input and high-input trials.

Today, development and selling of wheat seeds is done by a few large companies like KWS and Limagrain (KWS, 2021; Limagrain, 2021) and several smaller ones as well as public breeding programs, for example Agroscope in Switzerland (Agroscope, 2021). Some breeders only produce organic seeds, like Getreidezüchtung Peter Kunz or Cultivari gGmbH (GZPK, 2021; Cultivari, 2021), others produce both organic and conventional seeds, for example Saatzucht Donau GesmbH & Co KG (Saatzucht Donau, 2021). Currently, new organic cultivars are also being developed by two projects, LIVESEED and ECOBREED, both funded by Horizon 2020, an EU research program (Grausgruber et al., 2019; Weinhappel, 2018).
LIVESEED consists of 49 partner organizations from 17 EU member states and Switzerland. Its aims are to improve the sustainability, performance and competitiveness of organic agriculture. This is achieved by boosting organic seed production, developing new breeding approaches and by harmonizing the implementation of the European regulations in relation to

organic seed. LIVESEED analyzes the determining factor of the current production and use of organic seed, identifies breeding gaps and increases transparency of the EU organic seed market. The project involves several work packages, like increasing the quality and volume of organic seeds by using cultivars suitable for organic farming, sharing of knowledge and holding training courses across countries as well as investigating new seed health strategies and technologies with focus on the vitality of organic seed (Weinhappel, 2018).

ECOBREED is a project coordinated by the Agricultural Institute of Slovenia with collaboration of 25 partner organizations from 15 countries, including for example Austria, China, Germany, Poland, Romania, the United Kingdom and the United States. The main goal of ECOBREED is the improvement of the availability of seeds and varieties suitable for organic and low-input agriculture for wheat, potato, soybean and common buckwheat. The project wants to increase breeding activities and develop breeding material with improved stress resistance, efficiency of resources and quality. For wheat, the focal point lies on bunt resistance by marker-assisted selection (Grausgruber et al., 2019).

2 Research questions

This master thesis consists of two segregating populations of recombinant inbred (RI) winter wheat lines. One population was derived from a cross between PI166910 (resistant to common bunt) and ‘Rainer’ or ‘Lukullus’ (susceptible cultivars), respectively, the other from a cross between 702-1102C (common bunt resistant) and ‘Rainer’. The resistant parent PI166910 putatively harbors common bunt resistance genes *Bt7*, *Bt9* and *Bt11*, 702-1102C putatively carries *Bt8* and/or *Bt9*. This leads to different resistance levels towards common bunt in the offspring.

The aims of this master thesis can be roughly divided into two parts: phenotypic scoring and genotypic screening of the RIL populations. More specifically defined research questions for each part are as follows:

- Phenotypic scoring in the field:
    - How many and which lines are resistant to common bunt?
    - Which segregation pattern for common bunt severity is evident in each of the tested populations?
    - Does plant height have an influence on the resistance level?
    - Are heading dates crucial for the level of common bunt resistance?
    - Is there a phenotypic correlation between common bunt severity and brown rust severity?
    - Do genotypes with or without awns differ regarding common bunt susceptibility?

- Genotypic screening in the laboratory:
    - Are any of a selected set of KASP or SSR markers predictive for bunt resistance in one or both of the evaluated populations?
    - Do phenotypically resistant lines harbor one or two resistance genes (either *Bt9* on chromosome 6D or *Bt11* putatively on chromosome 3B or both) inherited from the resistant parent PI166910?
    - Is the population descending from 702-1102C segregating for *Bt8* or *Bt9* or both resistance genes?

3 Material and Methods

The following chapter gives information on plant material, field site and trait assessment. Details on genotyping of both mapping populations as well as on statistical analysis will be provided.

3.1 Plant material

Five recombinant inbred line (RIL) populations (Table 1) were derived from crosses between resistant (PI166910, 702-1102C) and susceptible ('Rainer', 'Lukullus') winter wheat (*Triticum aestivum subsp. aestivum*) genotypes resulting in a total of 494 $F_{5:7}$ recombinant inbred lines. The five RIL populations can be grouped into two mapping populations based on the resistant parents (PI166910 and 702-1102C):

- PI166910 is a Turkish landrace (wheat gateway, s.a.) and carrier of bunt resistance gene *Bt11* (Goates et al., 2012) and probably *Bt9*. SSR markers indicative for the QTL interval of *Bt11* on chromosome 3B have already been published (Ciucă, 2011). PI166910 also carries *Bt7* (GRIS, 2017), however, until completion of this thesis there were no markers available for this gene and therefore its presence could not be checked in the populations.
- 702-1102C, an experimental line developed by Erik Tybirk (Denmark) carries either *Bt8* or *Bt9* (Anders Borgen, personal communication). Steffan et al. (2017) mapped *Bt9* to the long arm of chromosome 6D, which could be tested with KASP markers.
- 'Rainer' and 'Lukullus' are winter wheat cultivars registered in Austria by Saatzucht Donau GesmbH & CoKG, 'Rainer' in 2006 and 'Lukullus' in 2008 (Saatzucht Donau, s.a.). Both are highly susceptible to common bunt.

Table 1. *Information on RIL populations tested for common bunt resistance; SP = susceptible parent.*

Mapping population	RIL population	Individuals per RIL population	Female*Male	Putative resistance genes
PI166910*SP	U10	120	Rainer*PI166910	*Bt 7,9,11*
	U11	120	PI166910*Rainer	*Bt 7,9,11*
	U15	160	PI166910*Lukullus	*Bt 7,9,11*
702-1102C*SP	S21	49	702-1102C*Rainer	*Bt 8 or 9*
	P108	45	702-1102C*Rainer	*Bt 8 or 9*

3.2 Field experiment

This section provides information on inoculation and cultivation of seeds as well as trait assessment including common bunt incidence.

3.2.1 Common bunt inoculum preparation and inoculation

For artificial seed inoculation common bunt teliospores were harvested from infected spikes collected in the field trials in Tulln between 2015 and 2018. For this purpose, bunt balls extracted from the infected spikes were manually crushed with a pestle and then sieved two consecutive times with 500 µm and 125 µm Retsch test sieves to remove any parts of wheat heads and hulls of bunt balls. Cleaned teliospores were collected and stored in lockable glass containers until inoculation.

Inoculation of seeds was performed according to a modified procedure described by Goates (1996) using an aqueous methylcellulose solution (0.05 %) containing the harvested common bunt teliospore mix (mix of equal amounts of spores from all years). Methylcellulose (ROTH Nr 8421.2, M ~ 3000 g/mol, 3300-4500 mPa s) was dissolved in water (2 g/l) overnight at room temperature. A suspension was created by weighing 90 g of common bunt teliospores into an Erlenmeyer flask and filling up the flask to a volume of 300 ml with methylcellulose solution. This mixture was stirred until homogeneity was reached, resulting in a viscous suspension with a teliospore concentration of 0.3 g/ml. Seed samples of 20 g each were transferred into a small container and 0.6 ml of inoculum were pipetted onto the seeds using an Eppendorf dispenser-pipette. The containers were sealed and mechanically shaken to achieve even distribution of inoculum on all grains. Throughout the inoculation process the solution was magnetically stirred to prevent the spores from sedimentation. After inoculating the seeds, they were dried at room temperature for a few days and stored until cultivation.

3.2.2 Cultivation

Sowing of 577 previously inoculated seed samples of all lines in the five RIL populations and their parental lines as well as 15 lines from the common bunt differential set and 34 cultivars and lines from breeding programs was done on November 16th, 2018. Additionally, five lines and cultivars (Sel500, ‘Tillexus’, ‘Tilliko’, ‘Rainer’ and ‘Alessio’) were inoculated with an isolate collected from spikes of the cultivar ‘Tilliko’ in 2018, which was more aggressive than the “housekeeping”-isolate used for inoculation of the remaining lines. These specific lines were not part of this trial, however, they will be included in chapter 6 (Conclusion and Outlook). Field trials comprised two replications.

The experimental site was located near Tulln in Lower Austria at a latitude of 48°18'631'', a longitude of 16°03'133'' and 178 meters above sea level (Land Niederösterreich, 2021). During the growing period of 2018/19, the temperature in Tulln was normal (BOKU, 2021) in comparison to the 30-year mean measured in Langenlebarn (ZAMG, 2021). Precipitation during that time was mostly average compared to the 30-year mean, except for months May and June (Figure 3). The soil type at the field site is moist black earth (BFW, 2021).

Plots were arranged as double rows of 160 cm length with a distance of 33 cm between rows and 50 cm to the next plot. On November 16th, 2018, wheat lines and cultivars for this trial were sown. For each plot, 10 g of seeds were used. Weed management and fertilization was performed according to Table 2 and Table 3. The preceding crop on this site was maize (*Zea mays*).

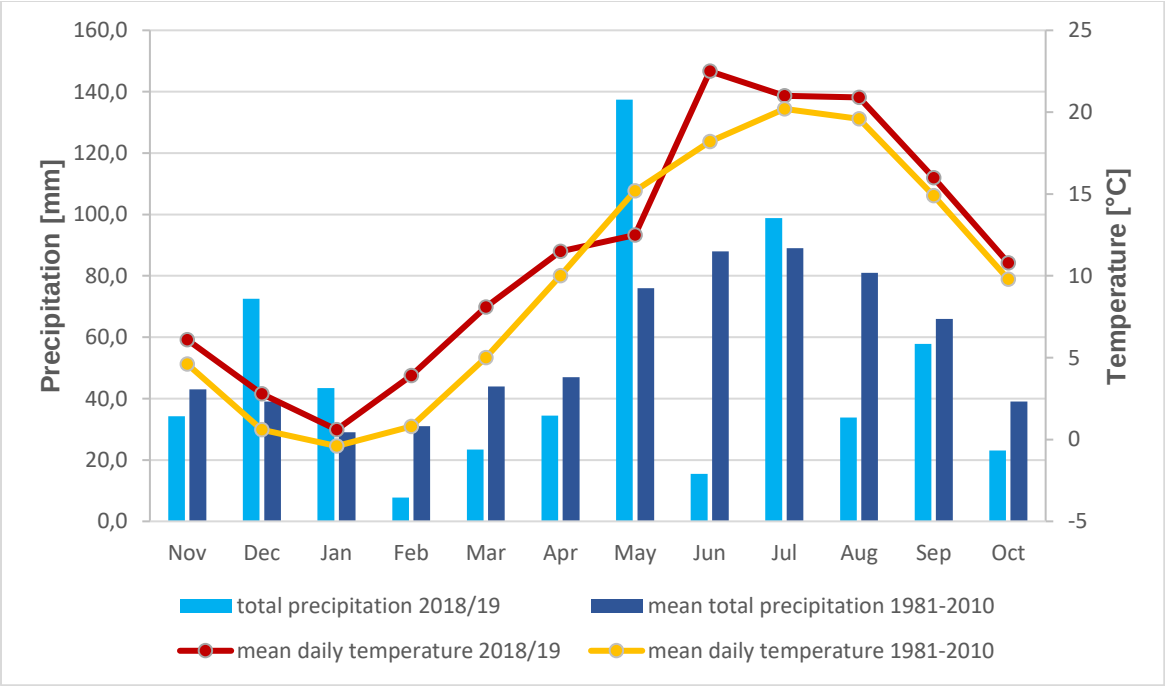

Figure 3. *Monthly precipitation and mean daily temperatures in Tulln, Austria during the growing period 2018/19 (November 1st, 2018 to October 31st, 2019) compared to long-term precipitation and temperatures in Langenlebarn, Austria between 1981 and 2010.*

Table 2. *Herbicides used, their application rates, manufacturers and active ingredients on the experimental fields in 2019 (source: Matthias Fidesser, personal communication).*

Date	Name	Application rate	Manufacturer	Active ingredients
08.04.2019	Andiamo Flexx	1.35 l/ha	Bayer Austria Ges.m.b.H.	Mecoprop-p, Diflufenican, Florasulam (Bayer Austria Ges.m.b.H., 2020)
28.05.2019	Express SX + Pixxaro EC	25 g/ha + 0.25 l/ha	Kwizda Agro	Tribenuron-Methyl, Halauxifen-methyl, Fluroxypyr (Kwizda Agro, 2021)

Table 3. *Fertilizers used, their application rates, suppliers and active ingredients on the experimental fields in 2019 (source: Matthias Fidesser, personal communication).*

Date	Name	Application rate	Supplier	Active ingredients
12.10.2018	NPK 0:15:40	285 kg/ha	Raiffeisen Ware Austria AG	15 % P_2O_5, 40 % K_2O
21.03.2019	NAC (Nitrate-Ammonium-Calcium)	250 kg/ha	Raiffeisen Ware Austria AG	27 % N
21.05.2019	NAC (Nitrate-Ammonium-Calcium)	160 kg/ha	Raiffeisen Ware Austria AG	27 % N

3.2.3 Common bunt assessment

Common bunt incidence was determined by counting infected wheat heads in each field plot. Scoring started in late June of 2019 and was finished in the middle of July of the same year. In each plot, 150 heads were cut along the diagonal to detect bunt balls which contain common bunt teliospores (Figure 4). Initially, 75 heads of the first row of the double-row plot were assessed. If none of those heads were infected, a total common bunt incidence of zero was assumed. Given there was at least one bunted head in the first row, 75 heads of the second row were evaluated as well, resulting in a total of 150 heads. Common bunt incidence was expressed as the percentage of infected heads in the total number of assessed heads. If there

were less than 150 heads present in one plot and common bunt incidence was detected, all available heads were cut and an equivalent ratio was calculated.

Figure 4. *Assessment of common bunt infection: infected head (A); cut heads – infected (bottom) and healthy (top) (B).*

3.2.4 Assessment of traits besides common bunt

Date of heading. Date of heading was documented as the day when at least 50 % of a plot had reached BBCH 55. It was assessed approximately every second day (May 24th, 2019 until June 12th, 2019) and scored as days after the 1st of May.

Plant height. Plant height was determined once for each plot after the growing period. The average plant height per plot was measured from the ground up to the tip of the heads (without awns) in intervals of five centimeters.

Brown rust infection. Infection with brown rust (*Puccinia triticina*) was visually assessed as the percentage of infected leaf surface area on each plot.

Awns. After all plots had reached BBCH 59 (end of heading), the presence or absence of awns was determined. A plot was scored as “0” when none of the heads had awns, “1” when all heads expressed awns and “0.5” for heterogenous lines.

3.3 Genotyping RIL populations using KASP and SSR markers

Before lines of RIL populations could be analyzed with molecular markers, DNA had to be extracted from harvested leaves.

3.3.1 Preparation of samples

At the end of May 2019, ten leaves from each plot of the first replication of the field trial were harvested for DNA extraction. Before the leaf samples were placed in paper bags, their tips were cut off. Subsequently the samples were put in a drying chamber at 36 °C for five days and then stored at 4 °C until DNA extraction was performed.

3.3.2 DNA extraction

DNA extraction was carried out following the protocol used at the Institute of Biotechnology in Plant Production at IFA Tulln (see appendix, chapter 7.2) and applying some slight modifications. In a first step 1.2 ml 8-strip-tubes for each sample were labelled, placed in a 96-well rack and each tube was filled with three glass beads. Each sample of dried leaves was cut into an individual tube (up to a volume of 0.5 to 0.6 ml) using scissors and a funnel. To avoid contamination, instruments were cleaned with a paper towel after every sample. Filled Eppendorf tubes were stored at 4 °C until all samples were cut. Before DNA extraction, the previously cut leaves were ground in a Retsch-mill for ten minutes. DNA extraction was performed according to a modified procedure described by Saghai-Maroof et al. (1984). 650 µl CTAB-buffer (Table 4) were added to each tube and the racks were placed in a water bath with gentle rocking at 65 °C for 60 to 90 minutes.

Table 4. *Components of CTAB-buffer for DNA extraction.*

Stock concentration	Final concentration	100 ml (for one 96 well plate)
dH_2O		65 ml
1 M Tris - (pH 7.5)	100 mM	10 ml
5 M NaCl	700 mM	14 ml
0.5 M EDTA - (pH 8.0)	50 mM	10 ml
CTAB	1 %	1 g
14 M BME	140 mM	1 ml

Tubes were allowed to cool down to room temperature before adding 320 µl of chloroform:isoamylalcohol (24:1) to each tube and shaking gently by inversion for ten minutes. After the samples were centrifuged in an Eppendorf centrifuge for ten minutes at maximum speed, the top aqueous layer (about 300 µl) of the tubes was pipetted into new, labelled tubes. 260 µl of isopropanol were added and the tube contents were gently mixed and centrifuged for eight minutes at low revolutions per minute (rpm). Then a DNA-pellet stuck to the bottom of each tube and the remaining liquid was poured off. To this pellet, 100 µl of washing solution

“Wash 1” (Table 5) were added, tube contents were gently mixed for five minutes, incubated for 30 minutes at room temperature and centrifuged at low rpm for eight minutes. The remaining liquid was poured off and the DNA-pellet stuck to the bottom of the tube. 100 µl of washing solution “Wash 2” (Table 6) were added, the tube contents were gently mixed by inversion for five minutes and centrifuged for eight minutes at low rpm. The liquid was poured off and the DNA-pellet stuck to the bottom of the tube. DNA-pellets were dried overnight in open tubes (covered with a tissue) and dissolved in 100 µl TE-8 buffer (solution of 98.96 % distilled water, 1 % Tris with pH 8.0 and 0.04 % EDTA with pH 8.0) for several hours at room temperature on the next day. Dissolved DNA was then stored at 4 °C for at least 24 hours to allow proper dissolving.

Table 5. *Components of washing solution “Wash 1” for cleaning of DNA-pellets.*

Components	**For 100 µl**
76 % EtOH	76 µl
16 % dH_2O	16 µl
8 % 2.5 M NaOAc	8 µl

Table 6. *Components of washing solution “Wash 2” for cleaning of DNA-pellets.*

Components	**For 100 µl**
76 % EtOH	76 µl
23 % dH_2O	23 µl
1 % 1 M NH_4OAc	1 µl

3.3.3 DNA quantification and quality control

After DNA was dissolved, randomly chosen samples from each extraction-plate were analyzed for quality and quantity on a photometer (Shimadzu BioSpec-nano UV-VIS Spectrophotometer) by reading optical densities (OD) at 230 nm, 260 nm and 280 nm wavelengths. If quality parameters like absorption ratios between different wavelengths and DNA quantity in those samples were within the acceptable range, 100 µl of each tube in a 96-well plate were transferred into the according well in a Greiner Bio-One GmbH CELLSTAR® 96-well plate using a multi-channel pipette. DNA in these plates was then collectively quantified with a BioTek Instruments Synergy HT Multi-Mode Reader, subsequently automatically diluted to 150 ng/µl with dH_2O using an Eppendorf epMotion 5075 pipetting robot and stored at 4 °C

in sealed photometer plates. If results were not within the acceptable range, those DNA samples would have had to be further diluted until quality parameters were satisfying. However, samples in this trial did not have to be diluted further, as all of them showed satisfactory quality parameters. Before DNA could be used for molecular marker analysis, it had to be manually diluted to 30 ng/µl using dH_2O. Samples not analyzed immediately were stored at 4 °C.

3.3.4 Marker assisted selection using KASP markers

Kompetitive allele specific PCR (KASP) markers allow bi-allelic scoring of SNPs (single nucleotide polymorphisms) and InDels (insertions and deletions) at specific loci. KASP genotyping assays consist of three components:

- KASP assay mix containing two allele-specific primers with unique tail sequences and one common reverse primer,
- KASP master mix containing two fluorescence resonant energy transfer (FRET) cassettes and
- template DNA with the target SNP (LGC, 2015).

3.3.4.1 Pre-testing of KASP markers for allelic differentiation in parental lines

Before KASP markers could be used for selection and identification of resistance gene *Bt9* on chromosome 6D, they had to be tested on parental lines to check if markers were polymorphic between the resistant and the susceptible parents. No published KASP markers for *Bt9* were available at the start of this project. Therefore, KASP markers were chosen based on their chromosomal location on chromosome 6D on the physical map using the Chinese Spring Reference Sequence IWGSC RefSeq V1 (International Wheat Genome Sequencing Consortium [IWGSC] et al., 2018) in the region where *Bt9* has been mapped with DaRT*seq* and SSR markers by Steffan et al. (2017). Parental PCR with KASP markers K135 to K142 (Table 8) was therefore conducted with PI166910, 702-1102C, ‘Lukullus’ and ‘Rainer’.

PCR-reactions (Table 7*)* were performed on a BioRad 384-well Hard-Shell PCR plate with eight different KASP markers (Table 8) for 33 lines including parental lines relevant for this thesis (702-1102C, ‘Rainer’ and ‘Lukullus’ were loaded twice, PI166910 was loaded four times) and non-template controls (NTC). Allele-calls for the respective SNP (single nucleotide polymorphism) were named according to the parental lines (PI-allele, 702-allele and R-allele).

Table 7. *Reagents for parental KASP-PCR for each marker.*

Components	Per sample	90 µl
Template DNA (30 ng/µl)	2.5 µl	-
Master mix	2.43 µl	87.5 µl
Primer mix	0.07 µl	2.5 µl

DNA samples of 2.5 µl were pipetted into a BioRad 384-well Hard-Shell PCR plate, centrifuged, covered with a seal and stored at 4 °C. Master mix[1] and primer mix for KASP markers were defrosted, vortexed and centrifuged. Primer mix was composed of 45 µl dH_2O, 30 µl common primer, 12 µl forward primer and 12 µl reverse primer. 87.5 µl master mix and 2.5 µl primer mix were mixed in a 2.5 µl Eppendorf tube by vortexing. From this mixture, 2.5 µl were added to each DNA sample in the BioRad 384-well Hard-Shell PCR plate using a multichannel pipette. This process was individually executed for each KASP marker (K135 to K142). The loaded plate was then covered with a BioRad Microseal, centrifuged and placed into an Eppendorf Mastercycler® pro 384 with which a PCR-program was run. After PCR-reaction was finished, the plate was removed from the thermocycler and fluorescent signals were read using a BioRad CFX384 TM Real-Time System.

[1] Master mix was purchased from LGC Genomics, therefore exact components are unknown.

Table 8. *Marker information for KASP markers used for testing polymorphism among the parental lines of the RIL-populations. Mega base pair information refers to the position in the Chinese Spring Reference Sequence IWGSC RefSeq V1(International Wheat Genome Sequencing Consortium [IWGSC] et al., 2018).*

Marker name	Internal name	Primer	Primer sequences	Chromo-some	Physical position in mega base pairs (Mbp)
wsnp_Ra_c13881_21836489	K135	Allele specific primer 1	GAAGGTGACCAAGTTCATGCTgcataatgagcccaatcatccttC	6D	409.344012
		Allele specific primer 2	GAAGGTCGGAGTCAACGGATTgcataatgagcccaatcatccttT		
		Common primer	ggaatacttCtgcacagggga		
Excalibur_c30035_368	K136	Allele specific primer 1	GAAGGTGACCAAGTTCATGCTcactggtccatgacacaacaG	6D	459.23759
		Allele specific primer 2	GAAGGTCGGAGTCAACGGATTcactggtccatgacacaacaA		
		Common primer	cacagatgcatgtgcgctc		
wsnp_BM137835D_Ta_2_1	K137	Allele specific primer 1	GAAGGTGACCAAGTTCATGCTcaccttcagcttgctaattcttaC	6D	460.469809
		Allele specific primer 2	GAAGGTCGGAGTCAACGGATTcaccttcagcttgctaattcttaT		
		Common primer	tggaatatttttGcctaggtacaaC		
wsnp_BM137835D_Ta_2_3	K138	Allele specific primer 1	GAAGGTGACCAAGTTCATGCTttttgctattgtttagttgcatgtG	6D	460.470085
		Allele specific primer 2	GAAGGTCGGAGTCAACGGATTttttgctattgtttagttgcatgtT		
		Common primer	ctgaaatttggaagagttcactca		
BobWhite_c12032_371	K139	Allele specific primer 1	GAAGGTGACCAAGTTCATGCTggattgaaaacatccgcAtcG	6D	460.570558
		Allele specific primer 2	GAAGGTCGGAGTCAACGGATTggattgaaaacatccgcAtcA		
		Common primer	ctatgggaagctaaacttttcgtat		
RAC875_c48570_361	K140	Allele specific primer 1	GAAGGTGACCAAGTTCATGCTgagcttttccttgtaaagaagcaC	6D	460.573005
		Allele specific primer 2	GAAGGTCGGAGTCAACGGATTgagcttttccttgtaaagaagcaT		
		Common primer	accaGaTacggtacaTAacagaG		
BobWhite_c13435_700	K141	Allele specific primer 1	GAAGGTGACCAAGTTCATGCTtCCggTaTGAAcAGTTcgttC	6D	471.008508
		Allele specific primer 2	GAAGGTCGGAGTCAACGGATTtCCggTaTGAAcAGTTcgttT		
		Common primer	gccagaagtacaaagcaccC		
CAP7_c2559_543	K142	Allele specific primer 1	GAAGGTGACCAAGTTCATGCTgccattgagtgtcatcttgaagC	6D	471.017889
		Allele specific primer 2	GAAGGTCGGAGTCAACGGATTgccattgagtgtcatcttgaagA		
		Common primer	tcctgcatgctgccagaG		

3.3.4.2 Marker analysis using KASP markers RAC875_c48570_361 and BobWhite_c13435_700

Genotyping of the lines with KASP markers RAC875_c48570_361 (K140) and BobWhite_c13435_700 (K141) was performed with extracted and diluted DNA (30 ng/µl) separately for both markers in BioRad 384-well Hard-Shell PCR plates (Table 9).

Table 9. *Reagents for KASP-PCR with a single marker on a 384-well plate.*

Components	Per reaction	960 µl (for 384 samples)
Template DNA 30 ng/µl	2.5 µl	-
Master mix	2.43 µl	933.12 µl
Primer mix	0.07 µl	15.36 µl

DNA samples of 2.5 µl were transferred from Greiner Bio-One GmbH CELLSTAR® 96-well plates into BioRad 384-well Hard-Shell PCR plates, centrifuged, covered and stored at 4 °C. After master mix and primer mix were defrosted, vortexed and centrifuged, 933.12 µl master mix and 15.36 µl primer mix were mixed in a 2.5 ml Eppendorf tube. Using a multichannel pipette, 2.5 µl of this mixture were added to the previously loaded DNA in the BioRad 384-well Hard-Shell PCR plate. Before the plate was put into an Eppendorf Mastercycler® pro 384, it was sealed with a BioRad Microseal and centrifuged. PCR was conducted in the same way as described in subsection 3.3.4.1. and the results were evaluated with a BioRad CFX384 TM Real-Time System. This process was performed with both KASP markers (RAC875_c48570_361 and BobWhite_c13435_700) for each genotype in all RIL-populations except sub-population U15.

3.3.5 Marker analysis using SSR markers

SSR markers (simple sequence repeats), also called microsatellite markers, were used to screen for resistance QTL *Bt11* on chromosome 3B.

3.3.5.1 Pre-testing of SSR markers for detection of polymorphisms in parental lines

SSR markers were initially tested on PI166910, 702-1102C, ‘Rainer’, ‘Lukullus’, ‘Midas’ and N3BT3D (a “Chinese Spring” nulli-3B-tetra-3D line which has no 3B chromosomes but four 3D chromosomes). SSR markers in this trial (Table 10) were selected because they are polymorph and located at multiple regions on chromosome 3B (GrainGenes, 2021). PCR was conducted to

amplify the microsatellite markers; polymorphisms of markers were then detected by polyacrylamide gel electrophoresis.

Table 10. *Marker information for SSR markers used for parental PCR of the four parental lines in the RIL-populations as well as Midas and N3BT3D.*

SSR marker	Primer	Primer sequences[2]	Chromosome
Barc68	forward	cgATgccAAcAcAcTgAggT	3B
	reverse	AgccgcATgAAgAgATAggTAgAgAT	
GWM533	forward	AAggcgAATcAAAcggAATA	3B
	reverse	gTTgcTTTAggggAAAAgcc	
GWM644	forward	gTgggTcAAggccAAgg	3B
	reverse	AggAgTAgcgTgAggggc	
WMC500	forward	ATAgcATgTTggAAcAgAgcAc	3B
	reverse	cTTAgATgcAAcTcTATgcggT	
WMC505	forward	AggggAggAAAAccTTgTAATc	3B
	reverse	AcgAccTAcgTggTAgTTcTTg	
WMC808	forward	TgAAccATcATcggAgcTTg	3B
	reverse	TTTTAgccgAAgTcAAAcATTgc	

For this purpose, 2 µl of parental DNA (30 ng/µl) were loaded on a BioRad 384-well Hard-Shell PCR plate; each sample was loaded twice per SSR marker. Master mix for the PCR (Table 11) was prepared and thereof, 10 µl were pipetted onto the DNA for each marker, respectively. The loaded plate was sealed with a BioRad Microseal, centrifuged and placed into an Eppendorf Mastercycler® pro 384, which carried out a touchdown program to perform PCR.

[2] Source: GrainGenes, 2021

Table 11. *Reagents in SSR master mix for a single marker.*

Components	Stock concentration	Final concentration	150 µl (12 samples)
PCR buffer (incl. 15 mM $MgCl_2$)	10 X	1 X	15 µl
dNTP mix	2 mM	0.2 mM	15 µl
R-primer (10 µM)	10 µM	0.2 µM	3 µl
F-primer (10 µM) [3]	10 µM	0.03 µM	0.45 µl
M 13-30 primer (10 µM) [4]	10 µM	0.18 µM	2.7 µl
Taq-enzyme (5 U/µl)	0.05 U/µl	0.05 U/µl	1.5 µl
ddH_2O	-	-	112.35 µl

3.3.5.2 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed to compare parental alleles to the desired resistant allele and to simultaneously identify polymorphisms of markers. A separating gel (12 % Polyacrylamide) was prepared according to Table 12. APS and Temed were added shortly before pouring the gel since these components cause polymerization to start immediately. The separating gel was poured between two glass plates (previously cleaned with water and 70 % ethanol) and had a size of 33 cm x 10 cm and a thickness of 0.4 mm.

After 30 minutes, when the gel had polymerized, a comb was set and the stacking gel (Table 13) was poured. After polymerization, the gel was placed in a chamber filled with TBE as a running buffer and the comb was carefully removed.

Before PCR products were loaded onto the gel, 2.5 µl of loading buffer (30 % glycerol, 70 % H_2O and a pinch of Bromphenol blue) were added to all samples in the BioRad 384-well Hard-Shell PCR plate to dye them blue. The plate was centrifuged, covered with a BioRad Microseal and shaken. After 2 µl of each sample were loaded into the slots of the gel, electrophoresis was carried out at 400 V, 150 mA and 100 W for two hours. The electrophoresis chamber was connected to a cooling system.

[3] F-primer had an M 13-30 sequence at the 5' end.

[4] M 13-30 sequence (CCCAGTCACGACGTTG) was labelled with a fluorescent dye at the 5' end (FAM or Cy5) for fragment detection on the gel using a Typhoon Trio fluorescence scanner (GE Healthcare).

Table 12. *Components of a separating gel (12 % polyacrylamide) with a size of 33 cm x 10 cm x 0.4 mm for polyacrylamide gel electrophoresis of parental SSR-PCR.*

Components	For one separating gel
10 x TBE (Tris-Borate-EDTA) buffer	3.5 ml
dH_2O	21 ml
Acrylamide (29:1, 40 %)	10.5 ml
APS 10 % (Ammonium persulfate fresh)	368 µl
Temed (Tetramethylethylendiamin)	18.2 µl

Table 13. *Components of a stacking gel for polyacrylamide gel electrophoresis of parental SSR-PCR.*

Components	For one stacking gel
10 x TBE (Tris-Borate-EDTA) buffer	250 µl
dH_2O	1.94 ml
Acrylamide (29:1, 40 %)	310 µl
APS 10 % (Ammonium persulfate fresh)	52.5 µl
Temed (Tetramethylethylendiamin)	2.7 µl

3.3.5.3 Analysis of SSR markers

When the electrophoresis was finished, the gel (still between glass plates) was placed into an image scanner (Typhoon Trio, GE Healthcare, USA). Absorption of FAM labelled fragments was measured at a wavelength of 488 nm and of Cy5 labelled fragments at a wavelength of 633 nm. The scan was then edited and the bands were transformed into digital codes (allele calls):

- “1” represented alleles typical for the bunt resistant parental lines,
- “2” described alleles typical for the bunt susceptible parental lines and
- “3” referred to heterozygous individuals.

3.4 Statistical analysis

The statistical programming language R version 4.0.3 (R Core Team, 2020) was used for data analysis and plotting.

3.4.1 Best linear unbiased estimates (BLUEs)

To adjust for differences in replication numbers for each genotype, best linear unbiased estimates (BLUEs) were calculated for all traits observed in the field. The function “remlf90” in the package “breedR” (Muñoz and Sanchez, 2021) was used to fit a mixed model of the form:

$$t_{ik} = \mu + g_i + b_k + e_{ik} \quad (1)$$

With t_{ik} being the estimate for the respective trait for genotype *i* in replication *k*, μ being the overall mean, g_i referring to the ith genotype, b_k representing the kth replication and e_{ik} describing the error term. The genotype was considered a fixed effect while replication was considered as random. All further analyses on phenotypic data were performed using BLUEs, except boxplots, linear models and the Wilcoxon rank sum test.

3.4.2 Data structure

Histograms and scatterplots of each observed trait were plotted to determine data distribution and to relate common bunt incidence to other assessed traits (date of heading, plant height, brown rust infection and presence or absence of awns).

Boxplots for common bunt incidence per allele call were created separately for each KASP marker (RAC875_c48570_361 and BobWhite_c13435_700) and each mapping population (PI166910*SP and 702-1102C*SP).

3.4.3 Phenotypic correlations

The Pearson correlation coefficient was determined for all trait combinations using BLUEs. This was done using the “cor.test”-command from the “stats” package (R Core Team, 2020) at a significance level of $\alpha = 0.05$.

3.4.4 Analysis of variance

Variance components for genotype (σ_g^2), replication (σ_{rep}^2) and error (σ_e^2) were determined for each trait (common bunt incidence, date of heading, plant height, brown rust incidence and presence or absence of awns) by fitting a linear model as

$$t_{ik} = \mu + g_i + b_k + e_{ik} \quad (2)$$

With t_{ik} being the respective variance component, μ denoting the overall mean, g_i referring to the ith genotype, b_k representing the kth replication and e_{ik} describing the error term. In this linear model all factors were fit as random.

3.4.5 Repeatability

Previously determined variance components were used to calculate the repeatability, also called intra-class correlation coefficient (ICC), for each trait with the following formula (Nakagawa et al., 2017):

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{r}}. \quad (3)$$

σ_g^2 refers to the variance component of the genotype, σ_e^2 represents the residual variance and r describes the number of replications of the field experiment. Heritability was not calculated, because the trial only comprised one year of field testing.

3.4.6 Allele effects of common bunt resistance genes

Mapping populations were analyzed using linear models to estimate common bunt incidence depending on the presence of the resistant or susceptible allele. The Wilcoxon rank sum test (also called Mann-Whitney U test) was applied to analyze if there was a significant difference between the mean common bunt incidence of cultivars carrying the resistant or the susceptible allele, respectively.

A linear model comprising the allele calls of KASP markers RAC875_c48570_361 and BobWhite_c13435_700 was used to estimate common bunt incidence across replications:

$$t_i = \mu + m_i + e \quad (4)$$

The common bunt incidence for all lines with a specific allele call is denoted by t_i, the grand mean for bunt incidence is represented by μ, m_i describes the allele call for the ith marker (either RAC875_c48570_361 or BobWhite_c13435_700) and e refers to the error term. Common bunt incidence is the response variable; the allele calls for each line represent the predictor variables. The Wilcoxon rank sum test is a non-parametric method applied to compare the mean bunt incidences of genotypes carrying resistant or susceptible alleles in one of the two SNP-positions investigated with the KASP-markers, respectively. This test was performed in R using the "wilcox.test"-function from the "stats" package (R Core Team, 2020) with the argument "alternative" set to "two.sided". The null (H_0) and alternative (H_1) hypotheses were formulated as

$$H_0: \eta_R = \eta_S \quad (5)$$

$$H_1: \eta_R < \eta_S \quad (6)$$

with η_R being the mean common bunt incidence of genotypes carrying the resistant allele and η_S describing the mean common bunt incidence of genotypes with the susceptible allele. The "wilcox.test"-function in R ranks common bunt incidences in each group (R = resistant allele and S = susceptible allele) from lowest to highest. Values of the ranks are summed for each group and the test statistic U is determined as:

$$U_R = n_R n_S + \frac{n_R(n_R+1)}{2} - \Sigma T_R \quad (7)$$

$$U_S = n_R n_S + \frac{n_S(n_S+1)}{2} - \Sigma T_S \quad (8)$$

Sample sizes for both groups are represented by n_R and n_S, ΣT_R and ΣT_S refer to the previously calculated sum of ranks and U_R and U_S are the Mann-Whitney statistics (test statistics) for both groups (Gravetter and Wallnau, 2017). The null hypothesis was rejected when the p-value returned by the "wilcox.test"-function was smaller than 0.05.

4 Results

The following chapter shows general data structure and statistical results as well as the outcomes of PCRs performed with KASP and SSR markers for each mapping population.

4.1 Data structure

In this section, the set of differential lines will be discussed before mapping populations PI166910*SP and 702-1102C*SP will be analyzed regarding data structure comprising common bunt incidence, date of heading, plant height and brown rust incidence.

A set of differential lines (Table 14) was included in the trial to check the virulence of the isolate. The line carrying resistance gene *Bt9* (M90387_(Bt9)_BG) showed low common bunt incidences of 3.33 and 5.33 %, respectively, whereas line carrying *Bt11* (M822123_(Bt11)_BG) showed 0 %. Lines carrying resistance genes *Bt2*, *Bt7* and *Bt13* expressed relatively high common bunt incidences ranging from 26 to 55.33 %.

Table 14. *Lines of the differential set with incorporated resistance genes and common bunt incidences for both replications.*

Line	Resistance gene	CBI [%] (rep. 1)	CBI [%] (rep. 2)	CBI [%] mean
Sel2092	*Bt1*	0.00	0.00	0.00
Sel1102	*Bt2*	55.33	42.67	49.00
Ridit	*Bt3*	6.00	4.00	5.00
CI1558B	*Bt4*	6.00	4.00	5.00
Hohenheimer	*Bt5*	0.00	0.00	0.00
Rio	*Bt6*	0.00	4.00	2.00
Xenos	*Bt7*	37.33	53.33	45.33
SEL500-77	*Bt7*	54.00	50.00	52.00
M822161	*Bt8*	2.67	2.00	2.34
M90387	*Bt9*	5.33	3.33	4.33
M822102	*Bt10*	2.67	1.33	2.00
M822123	*Bt11*	0.00	0.00	0.00
PI119333	*Bt12*	0.00	0.00	0.00
Thule-III	*Bt13*	26.00	30.00	28.00
PI173437	*BtP*	9.33	10.67	10.00

4.1.1 Mapping population PI166910*SP

Common bunt incidence is expressed as the percentage of infected heads in the total number of assessed heads and is visualized in a histogram showing high levels of resistance in mapping population PI166910*SP (Figure 5). More than half of the tested lines (290 out of 399 individuals) expressed low susceptibility between zero and 4.99 % common bunt incidence, only two lines showed a maximum incidence of 65.1 to 70 %. It can also be observed that data regarding common bunt incidence is not normally distributed but skewed to the right.

Plant height showed a minimum of 95 cm and a maximum of 140 cm (Table 15). The most frequently occurring plant height was between 115.1 and 120 cm (in 116 out of 399 individuals) (Figure 5).

Date of heading, scored as the days after May 1st, had occurred between May 26th and June 9th (Table 15) with a peak on June 3rd. On this day 111 lines had reached BBCH 55. Within five days 92.5 % of all tested lines had reached their date of heading (between June 2nd and June 6th) (Figure 5).

Brown rust incidence scored in the field trial was generally low, ranging from three to 47.5 % (Table 15) with a peak at 18 % (Figure 5).

In mapping population PI166910*SP, almost 74 % of all lines fully expressed awns.

4.1.2 Mapping population 702-1102C*SP

Progeny of mapping population 702-1102C*SP showed a relatively high level of common bunt resistance with 40.22 % of all lines (37 out of 92 lines) expressing zero to 4.99 % common bunt incidence. The maximum common bunt incidence was 58 % (Table 16). Through a histogram, it can be seen that this data is skewed to the right (Figure 6).

In this mapping population, plant height ranged between a minimum of 70 and a maximum of 110 cm (Table 16). More than half of all lines (51 out of 92) reached plant heights between 90.1 and 100 cm (Figure 6).

Date of heading of all lines of mapping population 702-1102C*SP occurred within six days, starting on June 2nd and ending on June 7th (Table 16), with a peak on June 5th (Figure 6).

Brown rust incidence was low, with a minimum of 4 and a maximum of 30 % (Table 16). 18 out of 92 lines reached an incidence between 12.1 and 14 % (Figure 6).

None of the lines in mapping population 702-1102C*SP had awns.

Table 15. *Estimates for variance components of genotype ($\sigma^2_{Genotype}$), replication ($\sigma^2_{Replication}$) and error (σ^2_{Error}) as well as repeatability estimates (H^2) for each trait. BLUEs for means, minima and maxima for* ***mapping population PI166910*SP*** *for all traits observed in the field: common bunt incidence (CBI), plant height (PH), date of heading (DOH) as the days after the 1st of May, brown rust incidence (BRI) and presence or absence of awns, whereby the number of lines with (1), without (0) awns or with a mixed type (0.5), respectively, is shown for the latter trait.*

	CBI [%]	**PH [cm]**	**DOH**	**BRI [%]**	**Awns**		
$\sigma^2_{Genotype}$	248.65	29.51	1.91	50.16	0.17		
$\sigma^2_{Replication}$	0.09	14.60	0.003	21.90	6.71e-7		
σ^2_{Error}	10.14	46.83	0.58	34.07	0.005		
H^2	0.98	0.56	0.87	0.75	0.99		
					0	**0.5**	**1**
PI166910*SP (n = 399)					88	16	295
Mean	8.01	118.40	34.39	21.50			
Min	0.00	95.00	26.00	3.00			
Max	69.00	140.00	38.50	47.50			
U10 (Rainer*PI166910, n = 120)					39	7	74
Mean	8.49	120.70	34.45	19.41			
Min	0.00	100.00	31.00	6.50			
Max	69.00	140.00	37.00	42.50			
U11 (PI166910*Rainer, n = 120)					49	8	63
Mean	8.92	115.60	34.49	21.28			
Min	0.00	97.50	31.50	6.00			
Max	65.00	135.00	37.00	47.50			
U15 (PI166910*Lukullus, n = 159)					0	1	158
Mean	6.96	118.80	34.28	23.24			
Min	0.00	95.00	26.00	3.00			
Max	65.67	137.50	38.50	47.50			

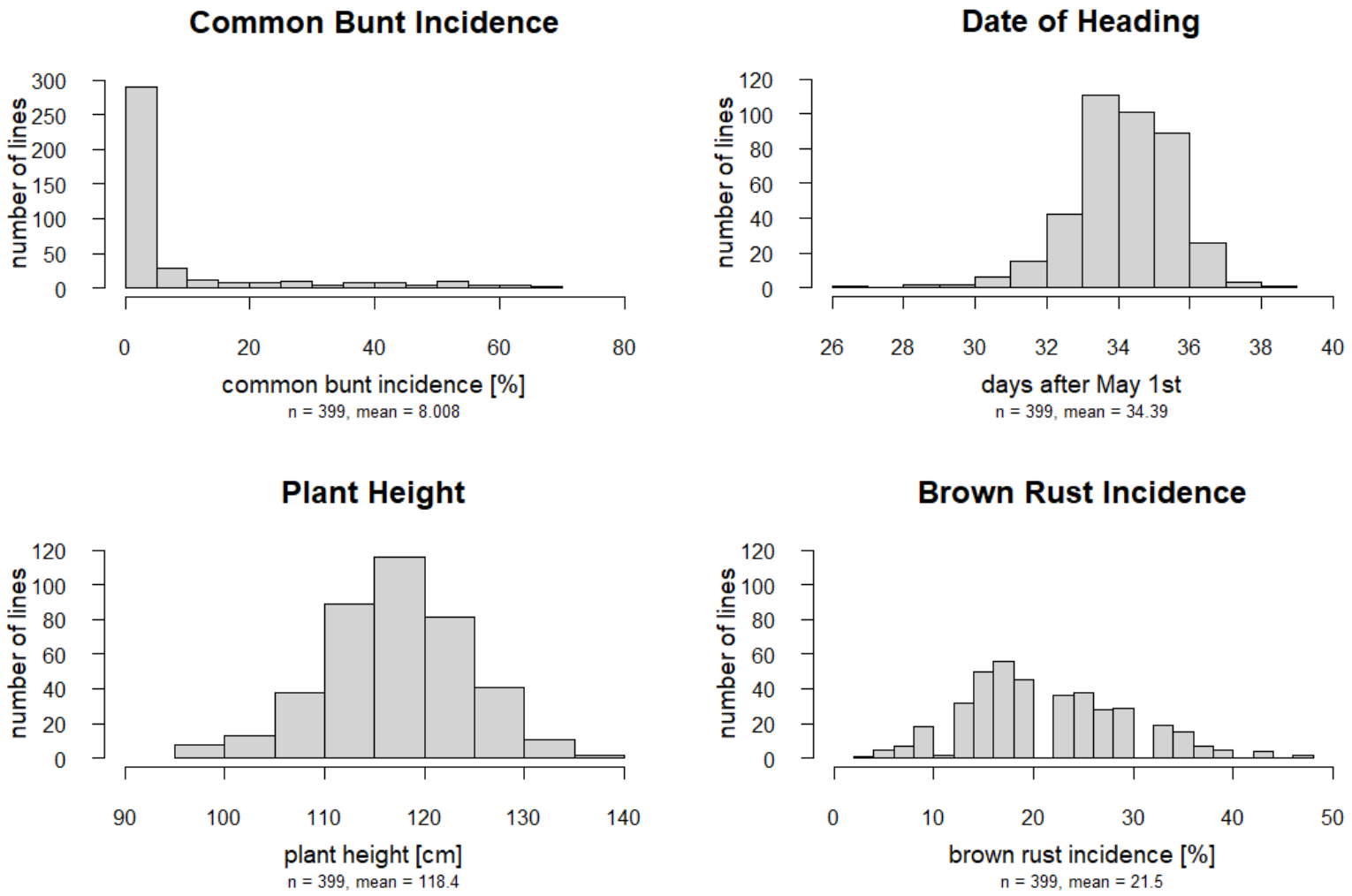

Figure 5. *Histograms showing the data distribution of the BLUEs for* ***mapping population PI166910*SP*** *for common bunt incidence, date of heading, plant height and brown rust incidence.*

Table 16. *Estimates for variance components of genotype ($\sigma^2_{Genotype}$), replication ($\sigma^2_{Replication}$) and error (σ^2_{Error}) as well as repeatability estimates (H^2) for each trait. BLUEs for means, minima and maxima for* ***mapping population 702-1102C*SP*** *for all traits observed in the field: common bunt incidence (CBI), plant height (PH), date of heading (DOH) as the days after the 1st of May, brown rust incidence (BRI) and presence or absence of awns, whereby the number of lines with (1), without (0) awns or with a mixed type (0.5), respectively, is shown for the latter trait.*

	CBI [%]	**PH [cm]**	**DOH**	**BRI [%]**	**Awns**		
$\sigma^2_{Genotype}$	229.00	30.65	0.98	17.03	-		
$\sigma^2_{Replication}$	0.39	3.46	0.01	26.58	-		
σ^2_{Error}	38.21	77.51	0.59	16.54	-		
H^2	0.92	0.44	0.77	0.67	-		
					0	**0.5**	**1**
702-1102C*SP (n = 184)					184	0	0
Mean	15.89	93.59	35.57	12.95			
Min	0.00	70.00	32.50	4.00			
Max	58.00	110.00	38.00	30.00			
S21 (702-1102C*Rainer, n = 96)					96	0	0
Mean	16.91	94.90	35.27	13.25			
Min	0.00	77.50	32.50	4.00			
Max	58.00	107.50	38.00	30.00			
P108 (702-1102C*Rainer, n = 88)					88	0	0
Mean	14.78	92.16	35.90	12.62			
Min	0.00	70.00	33.00	5.00			
Max	37.00	110.00	38.00	27.50			

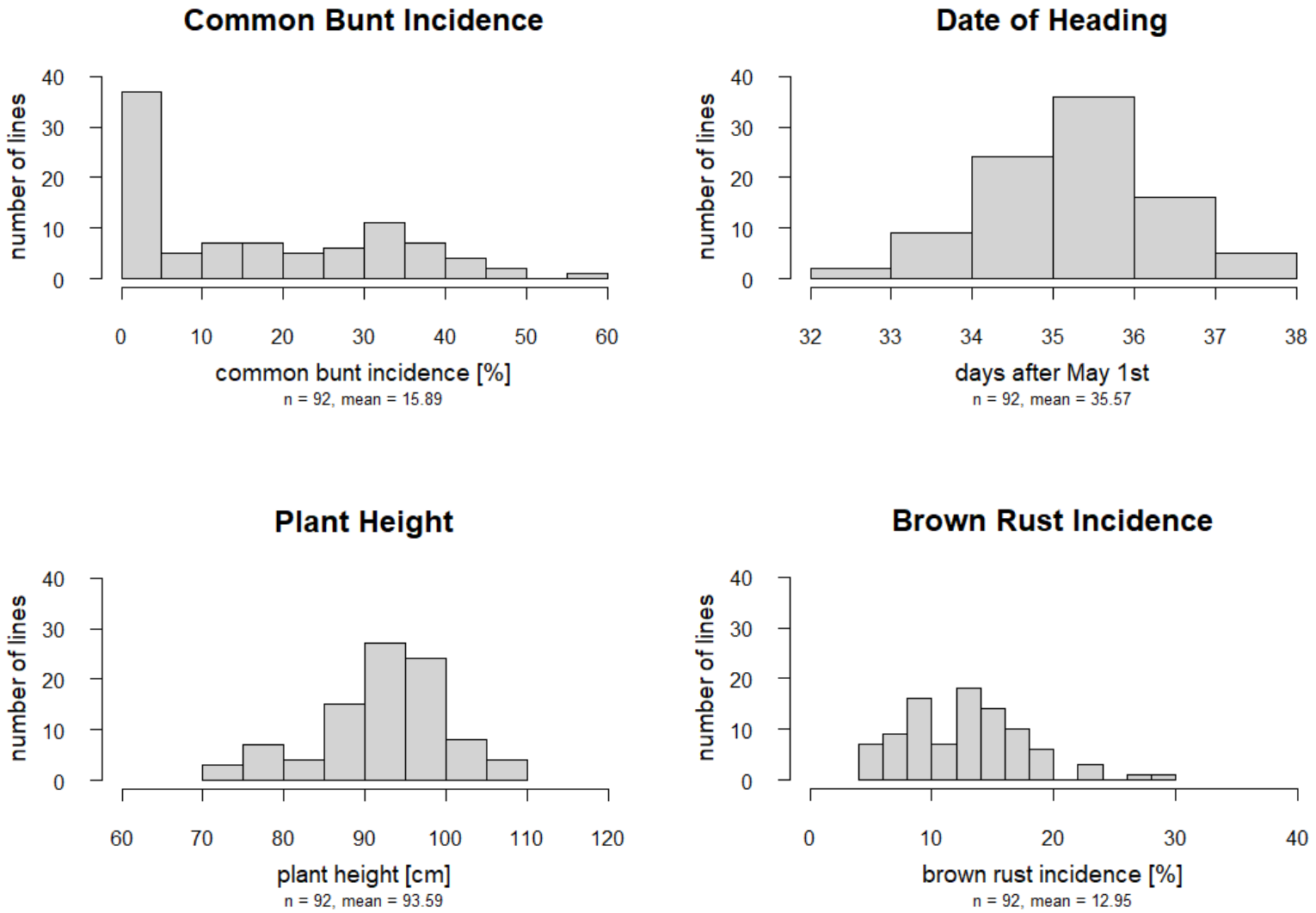

Figure 6. *Histograms showing the data distribution of the BLUEs for* ***mapping population 702-1102C*SP*** *for common bunt incidence, date of heading, plant height and brown rust incidence.*

4.2 Variance components

Variance components were calculated separately for each mapping population using a linear model (formula 2).

4.2.1 Mapping population PI166910*SP

Genetic variation related to common bunt incidence (CBI) was the largest variance component in mapping population PI166910*SP ($\sigma^2_{Genotype} = 248.65$) (Table 15). The error variance for CBI was $\sigma^2_{Error} = 10.14$. The replication variance continuously showed lowest values within the different variance components for each trait.

The genotype effect had the largest influence on all traits, except for plant height (PH) ($\sigma^2_{Genotype} = 29.51$). The error variance for PH showed the highest value compared to the error variances of other traits ($\sigma^2_{Error} = 46.83$).

Variance components for date of heading were perpetually low, ranging from $\sigma^2_{Replication} = 0.003$ to $\sigma^2_{Genotype} = 1.91$.

Variance in brown rust incidence scorings was also most strongly affected by the genotype ($\sigma^2_{Genotype} = 50.16$), but at the same time the error variance was also comparably high ($\sigma^2_{Error} = 34.07$).

Presence or absence of awns was the only trait with variance components in the narrow range between zero and one ($\sigma^2_{Replication} = 6.71e-7$ and $\sigma^2_{Genotype} = 0.17$).

4.2.2 Mapping population 702-1102C*SP

As already observed in mapping population PI166910*SP, the genotype effect for common bunt incidence (CBI) also accounted for the largest part of the total variance in mapping population 702-1102C*SP, with $\sigma^2_{Genotype} = 229.00$ (Table 16). The error variance for CBI was relatively high ($\sigma^2_{Error} = 38.21$).

Plant height showed the highest error variance with $\sigma^2_{Error} = 77.51$. Comparatively, the genotypic variance was relatively low with only $\sigma^2_{Genotype} = 30.65$.

Date of heading was the only trait with variance components between zero and one ($\sigma^2_{Replication} = 0.01$ and $\sigma^2_{Genotype} = 0.98$).

Brown rust incidence showed relatively high estimates for all three variance components, ranging from $\sigma^2_{Error} = 16.54$ to $\sigma^2_{Replication} = 26.58$.

For presence or absence of awns no variance components were calculated, as none of the tested lines in mapping population 702-1102C*SP had awns.

4.3 Repeatability

Repeatability estimates were derived from variance components (formula 3) and were calculated individually for each mapping population.

4.3.1 Mapping population PI166910*SP

In mapping population PI166910*SP, repeatability estimates for the observed traits were diverse (Table 17). Awns and common bunt incidence had the highest values ($H^2 = 0.99$ and $H^2 = 0.98$, respectively) while plant height showed the lowest repeatability with $H^2 = 0.56$. Brown rust incidence and date of heading had relatively high repeatability estimates of $H^2 = 0.75$ and $H^2 = 0.87$.

Table 17. *Repeatability estimates (H^2) for each trait observed on all lines of mapping population PI166910*SP.*

Trait	Repeatability
Common bunt incidence [%]	0.98
Plant height [cm]	0.56
Date of heading	0.87
Brown rust incidence [%]	0.75
Awns	0.99

4.3.2 Mapping population 702-1102C*SP

In mapping population 702-1102C*SP, most traits showed relatively low to medium repeatability estimates (Table 18). Common bunt incidence was the only trait with a high repeatability estimate ($H^2 = 0.92$). Of all tested traits, plant height showed the lowest repeatability with $H^2 = 0.44$. Brown rust incidence and date of heading had medium repeatability estimates ($H^2 = 0.67$ and $H^2 = 0.77$, respectively). For the trait presence or absence of awns, no repeatability was calculated, because no tested line in mapping population 702-1102C*SP had awns.

Table 18. *Repeatability estimates (H^2) for each trait observed on all lines of mapping population 702-1102C*SP.*

Trait	Repeatability
Common bunt incidence [%]	0.92
Plant height [cm]	0.44
Date of heading	0.77
Brown rust incidence [%]	0.67
Awns	-

4.4 Phenotypic correlations

For each mapping population, phenotypic correlations were calculated and corresponding scatterplots were created.

4.4.1 Mapping population PI166910*SP

Negative correlations between plant height and common bunt incidence as well as brown rust incidence and date of heading could be observed with Pearson correlation coefficients of $r = -0.24$ (Table 19). Brown rust incidence and plant height also showed a negative correlation with $r = -0.14$. Scatterplots visualize correlations between traits including regression lines in Figure 7.

Table 19. *Phenotypic correlations between all assessed traits except presence or absence of awns for mapping population PI166910*SP. Only correlations significant at α = 0.05 are shown, non-significant correlations are indicated as "n.s.". Corresponding p-values are given in parentheses.*

	CBI [%]	PH [cm]	DOH
PH [cm]	-0.24 (1.48e-6)		
DOH	n.s.	n.s.	
BRI [%]	n.s.	-0.14 (0.0058)	-0.24 (1.91e-6)

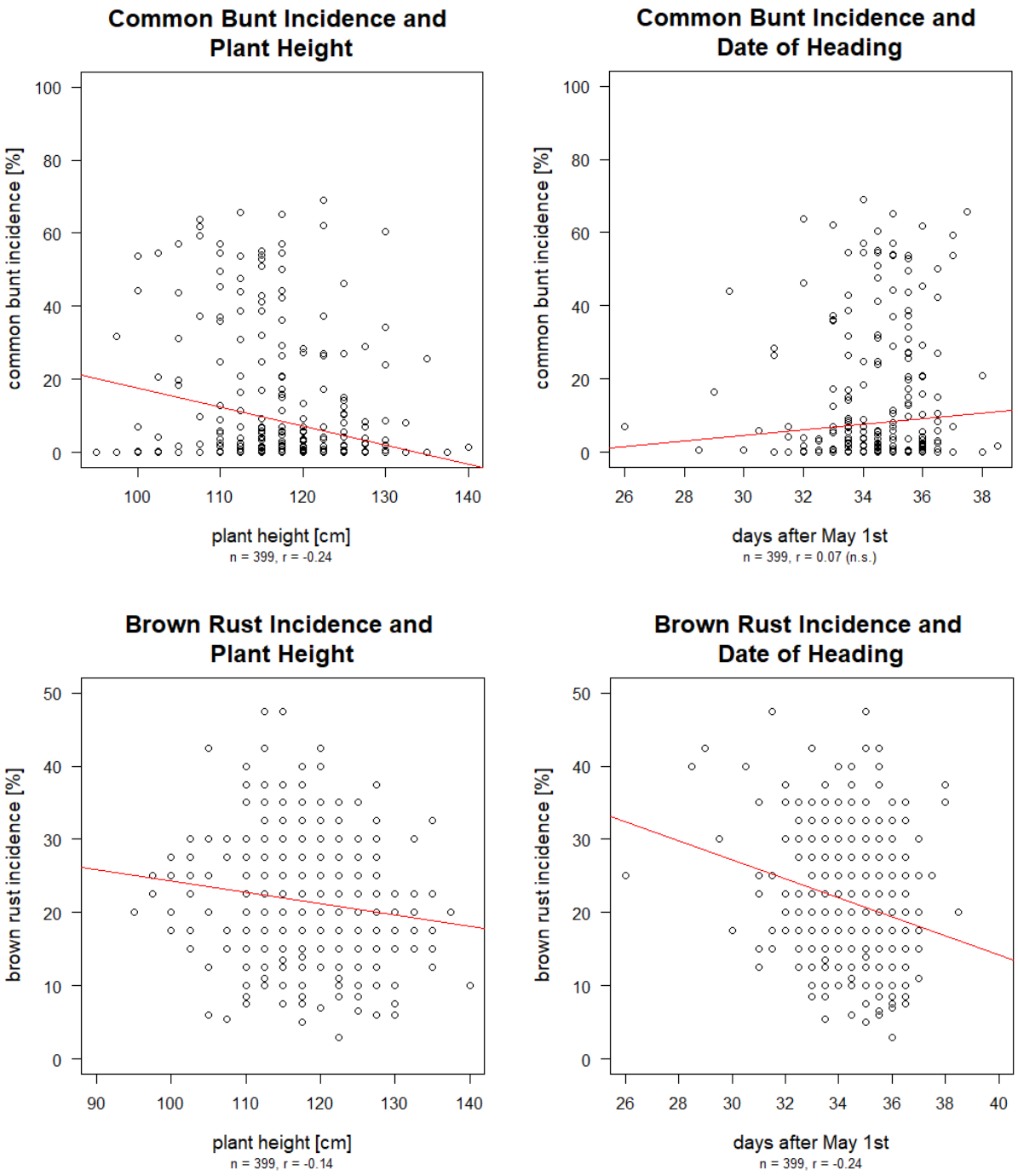

Figure 7. *Scatterplots with regression lines and regression coefficients showing the relationship between common bunt incidence and plant height, common bunt incidence and date of heading, brown rust incidence and plant height and brown rust incidence and date of heading. Data of BLUEs including lines of mapping population PI166910*SP is shown. "n.s." = non-significant.*

4.4.2 Mapping population 702-1102C*SP

In mapping population 702-1102C*SP, a low negative correlation could be observed between common bunt incidence and brown rust incidence with a Pearson correlation coefficient of $r = -0.10$ (Table 20). Phenotypic correlations between the remaining traits were non-significant. Scatterplots visualize selected correlations in Figure 8.

Table 20. *Phenotypic correlations between all assessed traits except presence or absence of awns for mapping population 702-1102C*SP. Only correlations significant at α = 0.05 are shown, non-significant correlations are indicated as "n.s.". Corresponding p-values are given in parentheses.*

	CBI [%]	**PH [cm]**	**DOH**
PH [cm]	n.s.		
DOH	n.s.	n.s.	
BRI [%]	0.23 (0.02497)	n.s.	n.s.

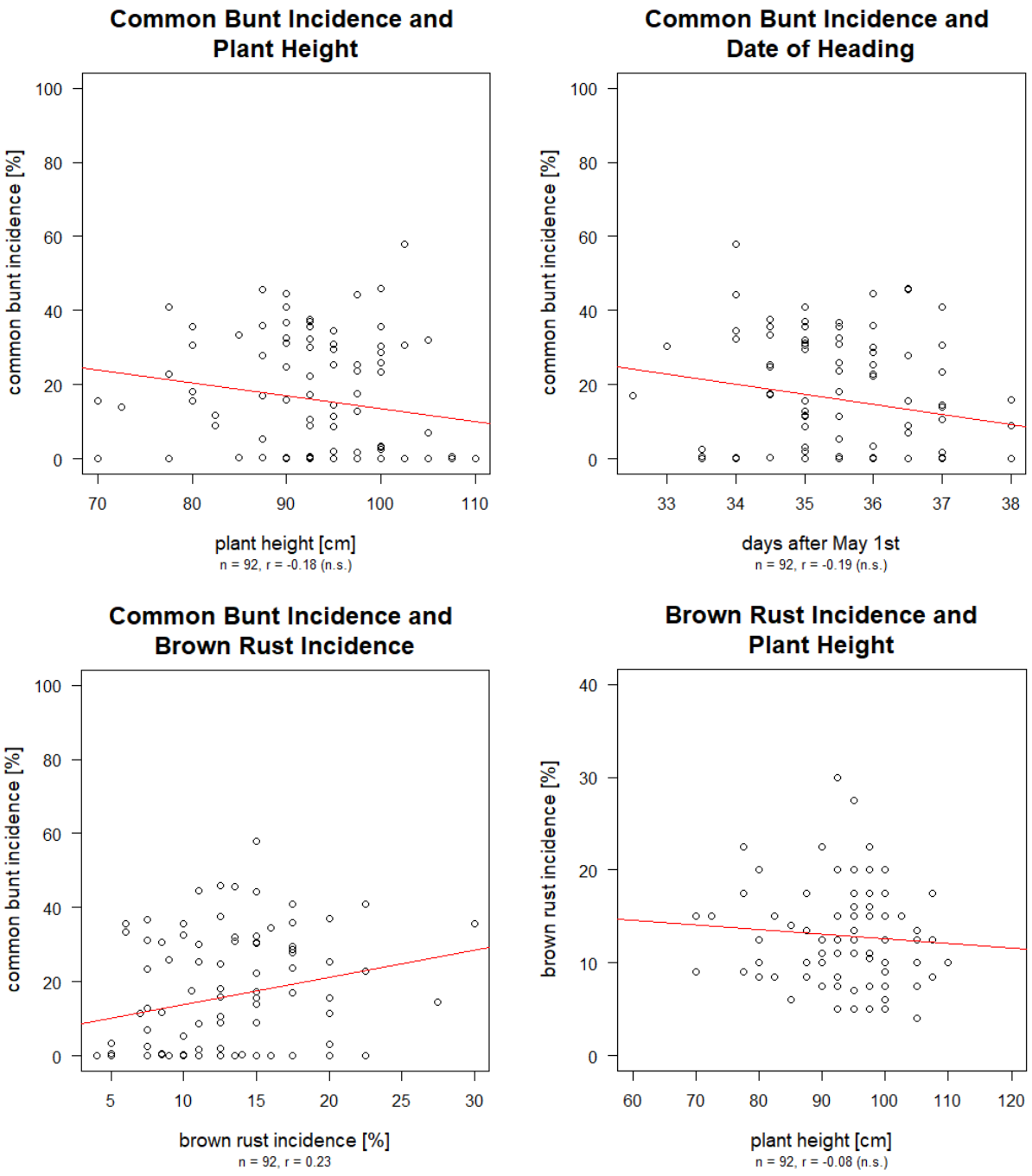

Figure 8. *Scatterplots with regression lines and regression coefficients showing the relationship between common bunt incidence and plant height, common bunt incidence and date of heading, common bunt incidence and brown rust incidence and brown rust incidence and plant height (top left to bottom right). Data of BLUEs including lines of mapping population 702-1102C*SP is shown. "n.s." = non-significant.*

4.5 Marker-trait associations

This chapter presents results of KASP and SSR analysis as well as results of linear models and Wilcoxon rank sum tests.

4.5.1 KASP markers

Initially, results of parental PCR are demonstrated followed by results of KASP markers for both mapping populations generated by screening for resistance gene *Bt9* on chromosome 6D.

4.5.1.1 Parental PCR

Parental PCR identified KASP marker **RAC875_c48570_361 (K140)** to show allelic differentiation between PI166910 and 'Rainer' (Table 21). Accordingly, KASP marker RAC875_c48570_361 (K140) was used for genotyping and marker-trait analysis in crosses Rainer*PI166910 (U10) and PI166910*Rainer (U11). However, RAC875_c48570_361 (K140) showed no allelic differentiation between PI166910 and 'Lukullus' – as they both carry the same SNP allele – as well as between 702-1102C and 'Rainer' (both sharing SNP allele G). Because of this lack of polymorphism, RIL populations U15, S21 and P108 could not be analyzed with marker RAC875_c48570_361 (K140). Hereinafter, for better understanding allele 1 for marker RAC875_c48570_361 (K140) will be called "R-allele" and allele 2 will be called "PI-allele" (Table 21).

Table 21. *Allelic differentiation in parental PCR for KASP marker RAC875_c48570_361 (K140) including the SNP (single nucleotide polymorphism) allele for each parental line.*

Parental line	Allele call	SNP	Name
PI166910	Allele 2	A	PI-allele
PI166910	Allele 2	A	PI-allele
702-1102C	Allele 1	G	-
702-1102C	Allele 1	G	-
Rainer	undetermined[5]	missing	-
Rainer	Allele 1	G	R-allele
Lukullus	Allele 2	A	-
Lukullus	Allele 2	A	-

[5] allele call was not possible

Parental PCR for KASP marker **BobWhite_c13435_700 (K141)** demonstrated allelic differentiation between resistant parental lines PI166910 and 702-1102C (both carry SNP allele A) and susceptible parent 'Rainer' which carries the SNP allele G (Table 22). Progeny from those crosses (U10, U11, S21 and P108) were therefore analyzed with marker BobWhite_c13435_700 (K141). Nevertheless, this marker also showed no allelic differentiation between PI166910 and 'Lukullus' (both share SNP allele G). Hence, analysis of RIL population U15 could not be conducted with any of the available KASP markers. For better understanding, allele calls for marker BobWhite_c13435_700 (K141) will be renamed according to the parental lines as "PI-allele", "702-allele" and "R-allele" (Table 22).

Table 22. *Allelic differentiation in parental PCR for KASP marker BobWhite_c13435_700 (K141) including the SNP (single nucleotide polymorphism) allele for each parental line.*

Parental line	Allele call	SNP	Name
PI166910	Allele 1	G	PI-allele
PI166910	Allele 1	G	PI-allele
702-1102C	Allele 1	G	702-allele
702-1102C	Allele 1	G	702-allele
Rainer	Allele 2	A	R-allele
Rainer	Allele 2	A	R-allele
Lukullus	Allele 1	G	-
Lukullus	Allele 1	G	-

4.5.1.2 Mapping population PI166910*SP

Analyses of mapping population PI166910*SP were done with KASP markers RAC875_c48570_361 (K140) and BobWhite_c13435_700 (K141). Because the RIL population U15 could not be analyzed with any KASP marker according to the results of the parental KASP PCR, the current section only refers to RILs of the sub-populations U10 and U11.

For marker **RAC875_c48570_361 (K140)**, genotypes carrying the PI-allele generally expressed low common bunt incidence (Figure 9). Nevertheless, there were some outliers with a maximum of 69 %. Lines carrying the R-allele showed both low and high levels of common bunt incidence with a maximum of 67 %. With a median of 1.67 % half of these lines showed a particularly low susceptibility. Lines categorized as "Heterozygote" showed a relatively high level of resistance.

Marker **BobWhite_c13435_700 (K141)** identified genotypes carrying the PI-allele to express a high level of resistance with a median of 0 % common bunt incidence (Figure 10). Lines with the R-allele showed a wide range of incidence levels with a median of 4.83 %. The highest common bunt incidence of lines harboring the R-allele was 69 %. Heterozygotes generally had high resistance levels with a median of 0.83 % common bunt incidence and only one RIL with 54.67 % common bunt incidence.

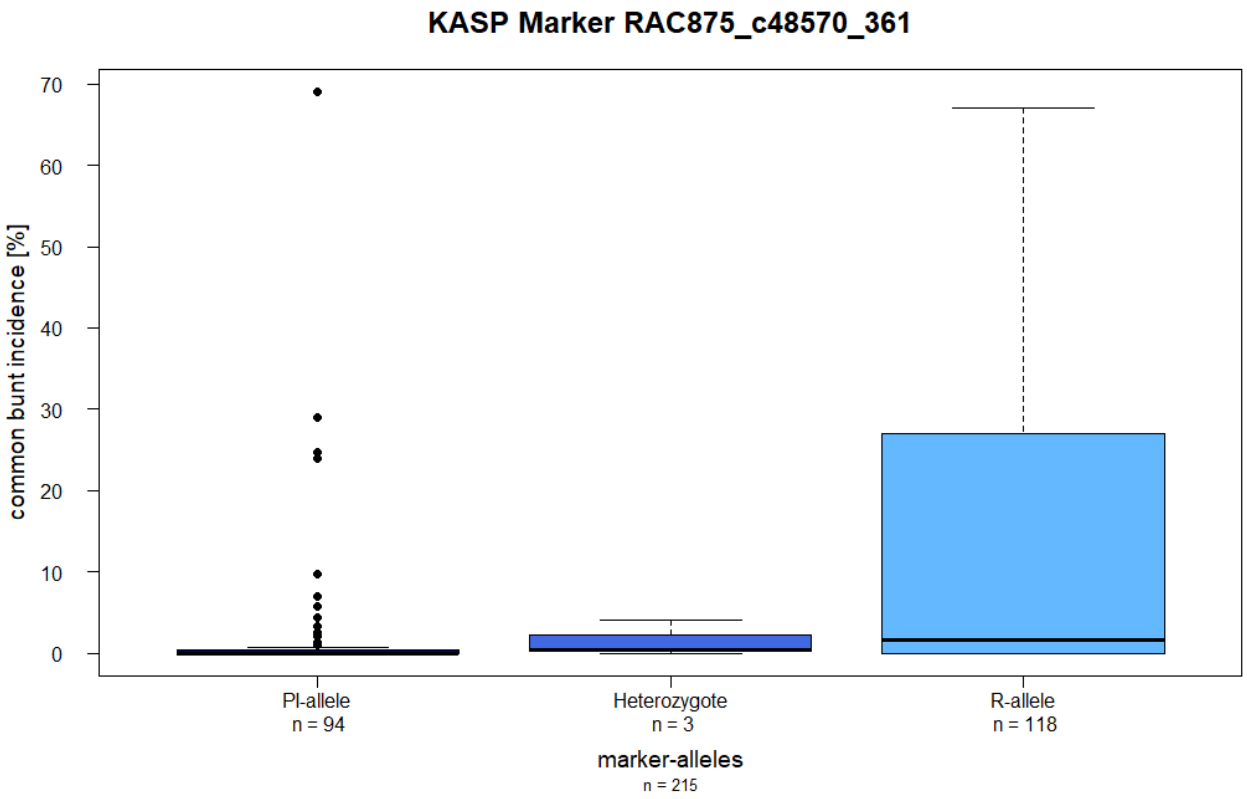

Figure 9. *Boxplot showing common bunt incidence [%] in RIL populations U10 and U11 for groups of lines with contrasting alleles for KASP marker RAC875_c48570_361 (K140) including the number of lines for each allele call.*

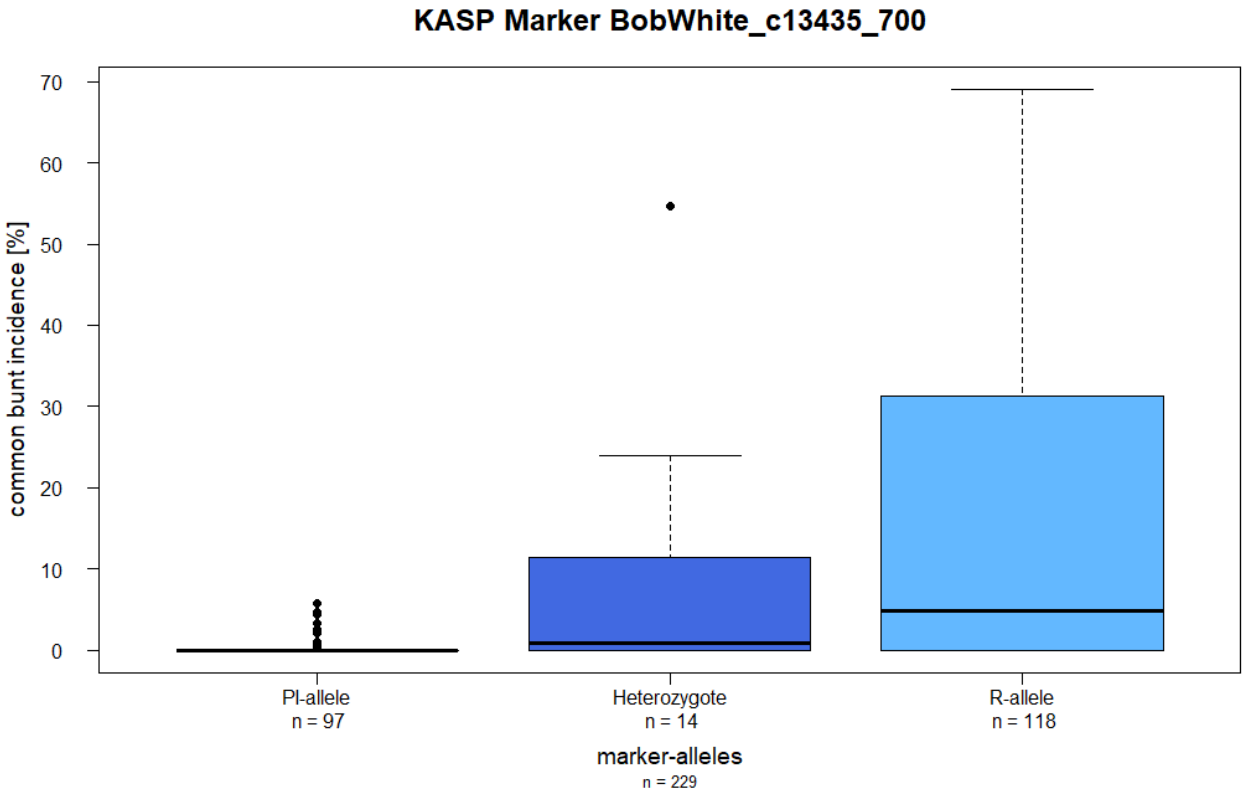

Figure 10. *Boxplot showing common bunt incidence [%] in RIL populations U10 and U11 for groups of lines with contrasting alleles for KASP marker BobWhite_c13435_700 (K141) including the number of lines for each allele call.*

A linear model built on data from KASP marker **RAC875_c48570_361 (K140)** as described in formula 4 showed that estimates of allele effects in terms of common bunt incidence were 12.15 % lower for lines carrying the PI-allele compared to those carrying the R-allele (p-value = 1.08e-7). The Wilcoxon rank sum test demonstrates that mean common bunt incidence levels of genotypes carrying the PI-allele or the R-allele, respectively, significantly differ from each other (p-value = 4.116e-9) which means that the null-hypothesis of no difference in mean infection levels between lines having contrasting alleles can be rejected.

Estimates of allele effects based on the linear model for KASP marker **BobWhite_c13435_700 (K141)** indicate that lines carrying the R-allele were 15.69 % more susceptible than lines carrying the PI-allele (p-value = 1.12e-11). Mean common bunt incidence levels of genotypes carrying the PI-allele or the R-allele, respectively, were significantly different as shown by the Wilcoxon rank sum test (p-value < 2.2e-16).

Mean common bunt incidences for lines carrying the PI-allele at both marker positions were analyzed and visualized with a histogram (Figure 11). Out of 94 lines carrying the PI-allele for marker K140 and 97 lines carrying the same allele for marker K141, 63 lines harbor the resistant allele for both markers. Thereof, most lines (87.3 %) showed very high resistance levels with zero to 0.5 % common bunt incidence, only one line expressed a maximum of 6 %.

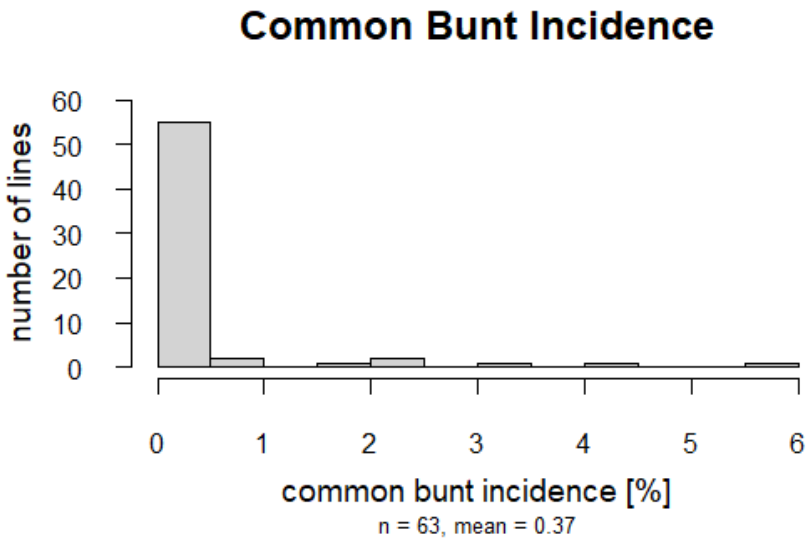

Figure 11. *Histogram showing mean common bunt incidence levels in lines with two resistance alleles from both replications of RIL populations U10 and U11 carrying the resistant allele for both markers RAC875_c48570_361 (K140) and BobWhite_c13435_700 (K141) located near resistance gene Bt9.*

4.5.1.3 Mapping population 702-1102C*SP

Mapping population 702-1102C*SP was analyzed using KASP marker **BobWhite_c13435_700 (K141)** which is illustrated in Figure 12 showing allele calls and common bunt incidences. Lines carrying the 702-allele were highly resistant whereas genotypes with the R-allele showed high variation in common bunt incidence levels with a median of 29.82 % and one RIL with a maximum of 67 %. Heterozygotes showed a relatively low median of 3.83 % but a maximum of 41 % common bunt incidence.

The linear model described in formula 4 for KASP marker **BobWhite_c13435_700 (K141)** shows that the effect of the R-allele was estimated to cause 27.42 % more common bunt incidence compared to lines with the 702-allele (p-value < 2e-16). The Wilcoxon rank sum test indicates that

mean common bunt incidence values of genotypes carrying the 702-allele and the R-allele are significantly different (p-value = 7.222e-14).

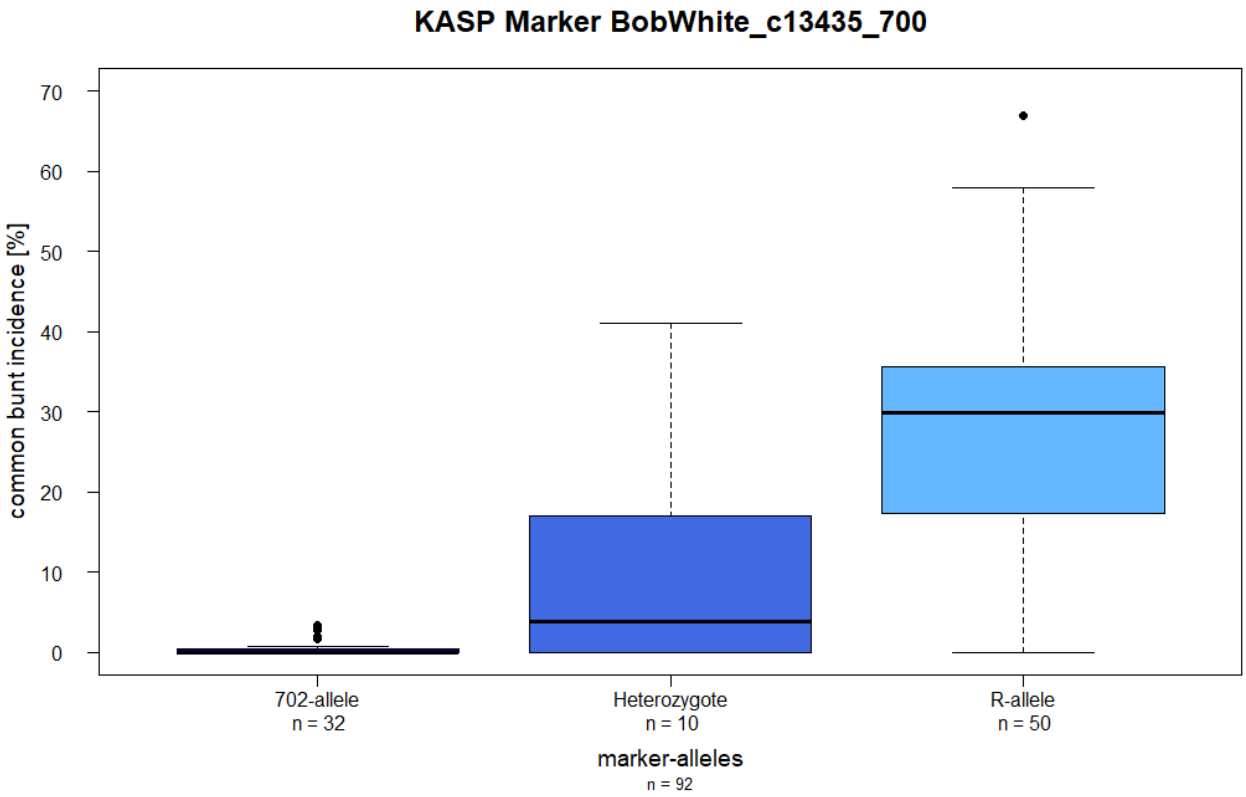

Figure 12. *Boxplot showing common bunt incidence [%] in mapping population 702-1102C*SP for groups of lines with contrasting alleles for KASP marker BobWhite_c13435_700 (K141) including the number of lines for each allele call.*

4.6 SSR markers

SSR markers were tested for the purpose of screening for resistance gene *Bt11* putatively mapping to chromosome 3B.

4.6.1 Parental PCR

Parental PCR and subsequent gel electrophoresis were performed with various SSR markers putatively mapping to chromosome 3B and therefore putatively linked to *Bt11* (Table 10). Nevertheless, none of the tested markers was polymorphic in the populations descending from PI166910 and 702-1102C (details not shown). Therefore, testing for the presence of resistance gene *Bt11* was not possible.

5 Discussion

This chapter discusses results presented in the previous chapter. Initially, the focus lies on the influence of individual traits of parental lines on their progeny; subsequently, the screening for common bunt resistance gene *Bt9* on chromosome 6D in the tested RILs will be debated.

5.1 Influence of traits of parental lines on progeny

The upcoming section addresses parental traits related to common bunt incidence as well as to other traits observed in mapping populations PI166910*SP and 702-1102C*SP.

5.1.1 Plant height

In 1991, Gaudet et al. suggested that a reduced plant height or stunting of more than 15 %, respectively, results in higher susceptibility levels of common bunt in wheat cultivars. Additionally, regardless of the degree of stunting, cultivars in taller height classes seemed to be more resistant than cultivars in shorter height classes in their experiments. However, this theory was disproved amongst others by Goates in 1996 as well as Singh et al. in 2016. It was discovered that there is a correlation between common bunt resistance and reduced plant height, which was found by Goates (1996) for certain resistance loci, stating the opposite to the original thesis of Gaudet et al. (1991). Therefore, breeders could be able to simultaneously incorporate multiple favorable genes for both reduced plant height and common bunt resistance (Singh, 2016). Nevertheless, diseased plants are usually of stunted growth, reducing their height by up to one third (Brandstetter and Weinhappel, 2011).

As expected, mapping population PI166910*SP showed a significant negative correlation between common bunt incidence and plant height ($r = -0.24$) (Table 19). These lines reached plant heights between 95 and 140 cm with a mean common bunt incidence of 8.01 % (Table 15). Lines of mapping population 702-1102C*SP were generally shorter (70 to 110 cm) with a mean common bunt incidence of 15.89 %. This population also shows a negative correlation between those two traits; however, it is not significant. The difference in resistance levels between both mapping populations may result from the different resistance genes incorporated in the parental lines (PI166910 harbors *Bt7*, *Bt9* and *Bt11*; 702-1102C harbors *Bt9*). According to the differential set (Table 14), *Bt7* is not effective, *Bt8* and *Bt9* show little and *Bt11* shows no common bunt incidence, which may lead to higher resistance levels in parental line PI166910 and therefore its

progeny. Nevertheless, scatterplots show that lines with favorable traits, i.e. high levels of common bunt resistance and short plant height, can be selected in both mapping populations (Figure 7 and Figure 8).

Repeatability estimates for plant height for mapping populations PI166910*SP and 702-1102C*SP were relatively low ($H^2 = 0.56$ and $H^2 = 0.44$, respectively) (Table 17 and Table 18). However, this estimate was expected to be higher because lines should have similar heights across both replications as the soil and surroundings of the experimental field could be described as uniform. Nevertheless, as plant height was not measured by one person alone but by groups of people, this possibly led to dissimilarities in the way of measuring and therefore to integrating an error into the data set and subsequently causing this unexpectedly low repeatability.

5.1.2 Date of heading

Date of heading of mapping population PI166910*SP occurred within 14 days, from May 26th to June 8th (Table 15). However, the number of days within which date of heading was observed varied significantly between RILs of different sub-populations. Lines of RIL population U15 started to reach their date of heading six days earlier than those of U10 and U11. This may result from the genetic influence of parental line ‘Lukullus’ which has an early to medium heading date in contrary to ‘Rainer’ which has a medium heading date according to the “Beschreibende Sortenliste” (descriptive list of registered cultivars, AGES, 2020).

Lines of mapping population 702-1102C*SP reached their heading date within only six days, starting on June 2nd (Table 16). In contrary to mapping population PI166910*SP, RILs of sub-populations S21 and P108 completed heading concurrently. This may be due to ‘Rainer’ being the susceptible parent of both sub-populations S21 and P108 whereas mapping population PI166910*SP has two different susceptible parents – ‘Rainer’ and ‘Lukullus’.

The phenotypic correlations between common bunt incidence and date of heading in both mapping populations are not significant. The scatterplots clearly show that there is a chance to select resistant lines in each maturity group (Figure 7 and Figure 8).

5.1.3 Brown rust incidence

Brown rust incidence in mapping population PI166910*SP was relatively high, reaching up to 47.5 %. Incidence levels were similar in all three sub-populations U10, U11 and U15 (Table 15).

Lines of mapping population 702-1102C*SP were more resistant towards brown rust than lines of mapping population PI166910*SP. They only expressed maximum incidence levels of 30 % (Table 16). This leads to the assumption that 702-1102C potentially has a higher resistance level towards brown rust, which was probably inherited by the progeny.
The phenotypic correlation between common bunt and brown rust incidence was non-significant for mapping population PI166910*SP, leading to the opportunity to select lines resistant to both common bunt and brown rust (data not shown). The correlation for mapping population 702-1102C*SP was significantly positive, however, there is also a chance to select lines resistant to both diseases (Figure 8).

Variance components for brown rust incidence showed relatively high values for replication and error variance in both mapping populations indicating a quick progression of the disease (Table 15 and Table 16). The individual replications were scored on different days (June 17th and June 19th) with weather conditions changing in between. On the day of the first scoring the maximum temperature was 25 °C while the following days were cooler and dry. On the second day of scoring the maximum temperature was more than 30 °C (Magdalena Ehn, personal communication). Generally, *Puccinia triticina* develops well with warm weather conditions during the day (around 25 °C) and cool temperatures during the night (below 15 °C) facilitating dew formation (Häni et al., 2008). Therefore, brown rust had very good conditions to develop between both scoring dates which could cause this relatively high variance component for the replication.

5.1.4 Presence or absence of awns

Lines of mapping population PI166910*SP vary significantly regarding having awns or not. In contrary to ‘Rainer’, parental lines PI166910 and ‘Lukullus’ have awns. Sub-populations U10 and U11 comprise lines with and without awns as well as heterogeneous types. U15, however, only has lines with awns (except for one mixed type) and a mean common bunt incidence 1.5 to 2.0 % lower compared to the mean of the other two U-sub-populations. This would lead to the assumption that lines with awns are more resistant towards common bunt, however, lines without awns also show high levels of resistance (Figure 13). Nevertheless, in this trial susceptible parental line ‘Lukullus’ showed 20 to 30 % less common bunt incidence compared to the susceptible parental line of U10 and U11, ‘Rainer’ (data not shown). Therefore, the genetic influence of ‘Lukullus’ could explain the differences in resistance levels between sub-populations.

Conclusively, according to Figure 13, resistant lines with any type of awns can be selected in mapping population PI166910*SP.
Lines of mapping population 702-1102C*SP did not express awns.

5.2 Screening for resistance gene *Bt9* using SNP markers

Common bunt resistance gene *Bt9* on chromosome 6D was putatively inherited from both resistance donors to all tested RIL populations. *Bt9* has been mapped to chromosome 6D by Steffan et al. (2017). Prior to the work on this thesis, the team of the Institute of Biotechnology in Plant Production at IFA Tulln searched for KASP markers near the mapped *Bt9* position, which were then used for marker analysis. SNP markers RAC875_c48570_361 and BobWhite_c13435_700 showed distinct allelic differentiations between the resistant and susceptible parents of all RILs of mapping population PI166910*SP, except for sub-population U15. These markers were therefore only used for testing sub-populations U10 and U11.
Results of KASP PCR of RIL populations U10 and U11 with marker RAC875_c48570_361 (K140) revealed that lines carrying the homozygous PI-allele expressed comparably high levels of resistance towards common bunt (Figure 9). However, several lines with the resistant allele at the location of this marker (460.573 Mbp) were still susceptible, which may be due to K140 being too far away from the resistance gene. One RIL carrying the resistant allele showed a maximum level of susceptibility of 69 % common bunt incidence. Lines carrying the R-allele as well as heterozygotes also showed relatively high levels of common bunt resistance. This may be due to PI166910 segregating for two resistance genes, *Bt9* and *Bt11*. Lines carrying only *Bt11* are probably also highly resistant explaining the high resistance levels of lines without the PI-allele. KASP marker BobWhite_c13435_700 (K141) demonstrated that lines of RIL population U10 and U11 homozygously harboring the PI-allele showed very high levels of resistance (Figure 10) with a maximum common bunt incidence of 5.67 % at this location (471.008 Mbp). Lines carrying the R-allele and heterozygous lines again showed low common bunt incidences putatively due to resistance gene *Bt11*. Analysis of lines carrying the PI-allele for both markers showed very high levels of resistance demonstrating that RAC875_c48570_361 and BobWhite_c13435_700 are probably indicative of *Bt9* (Figure 11).

For mapping population 702-1102C*SP only SNP marker BobWhite_c13435_700 (K141) showed allelic differentiations between the resistant and susceptible parents. Analysis of 702-1102C*SP with this marker revealed that lines carrying the homozygous 702-allele expressed very low levels

of susceptibility with a maximum of 3.33 % common bunt incidence (Figure 12). Based on Figure 12 it can be assumed that population 702-1102C*SP is not segregating for both resistance genes *Bt8* and *Bt9* but only for the latter, as all lines carrying the 702-allele express high levels of resistance whereas lines carrying the R-allele are mostly susceptible. If the population segregated for both resistance genes it would be expected that several lines carrying the R-allele are highly resistant.

These satisfactory results indicate that markers RAC875_c48570_361 (K140) and BobWhite_c13435_700 (K141) at 460.573 and 471.008 Mbp, respectively, are positioned already very close to the actual location of resistance gene *Bt9*. In this regard it is important to mention that Wang et al. (2019) mapped a QTL (*QDB.ui-6DL*) for dwarf bunt resistance to the long arm of chromosome 6D, ranging from 469.707 to 471.106 Mbp. KASP markers for this QTL were then designed and validated. They conclusively discovered that the 6D QTL is co-located with common bunt resistance gene *Bt9*. Thus, marker BobWhite_c13435_700 (K141) is located within this QTL and resistance gene *Bt9*, respectively.

To sum up, testing with marker RAC875_c48570_361 (K140) allowed for a relatively reliable distinction between carriers of resistant and susceptible alleles. However, marker BobWhite_c13435_700 (K141) showed even more precise results without outliers due to its closer location to the resistance gene. Therefore, it would suffice to analyze populations for resistance gene *Bt9* with SNP marker BobWhite_c13435_700 (K141) alone instead of both markers, thereby increasing the economic and time efficiency of marker analysis. However, the used KASP markers are only linked to *Bt9* and not diagnostic, as for instance SNP marker BobWhite_c13435_700 had the same allele ‘G’ in the resistant line PI166910 and the susceptible line ‘Lukullus’, and marker RAC875_c48570_361 showed the ‘A’ allele in PI166910 and ‘Lukullus’ and the ‘G’ allele in resistant line 702-1102C and susceptible cultivar ‘Rainer’. This is a typical finding when using linked markers. Ideally, markers that are in the causal gene and therefore detect alternative alleles would be preferable for breeding, but discovering such diagnostic markers is laborious and costly.

In this trial, 494 RILs were analyzed with KASP markers to screen for common bunt resistance gene *Bt9*. Compared to studies examining resistance genes against other diseases or traits in wheat, the number of lines in this study was high. Liu et al. (2020) used 67 CIMMYT breeding lines from different crosses and 56 NILs (near isogenic lines) to develop diagnostic markers (among others KASP markers) for the detection of wheat leaf rust resistance gene *Lr42* and to

validate their usefulness in breeding programs. Genotypic groups separated by the markers matched well with the obtained rust data of most lines. Zhang et al. (2021) aimed to identify QTL for FHB (Fusarium head blight) resistance in wheat using 236 $F_{5:7}$ RILs. Six FHB resistance QTL on five chromosomes were detected by constructing a linkage map with data derived from RIL populations. Populations in my trial, which potentially carry common bunt resistance gene *Bt11*, are being used for mapping and validation of this resistance gene. Wu et al. (2021) phenotyped 296 $F_{2:3}$ families to identify a candidate gene for hairy glume in wheat. The obtained data was used to develop a genetic linkage map for the target gene and to identify polymorphic KASP markers. In the course of this study three polymorphic KASP markers were found.

6 Conclusion and Outlook

Mapping populations PI166910*SP and 702-1102C*SP were phenotypically and genotypically characterized by field scoring and marker analysis. As expected, both populations expressed an adequate level of resistance. Although common bunt incidence is correlated with certain assessed traits, selection of lines with high resistance levels combined with other favorable traits is possible in each mapping population.

Recombinant inbred lines were screened with KASP markers in the course of this thesis. Evaluation of PCR analyses revealed that almost all lines expressing phenotypic resistance are very likely to harbor resistance gene *Bt9* inherited from parental lines PI166910 and 702-1102C based on allele calls at the investigated SNPs. According to the results, screening with SNP marker BobWhite_c13435_700 (471.008 Mbp) appears useful to satisfyingly analyze a population for the presence of *Bt9*. However, before this marker can be used for marker assisted selection, polymorphisms among the parents have to be evaluated, as marker BobWhite_c13435_700 is indicative but not diagnostic for *Bt9*. Further research for more tightly linked markers or diagnostic markers is therefore justified. Additionally, the population PI166910*SP could be genotyped with genomewide markers in order to have a chance to detect markers indicative of the highly effective resistance gene *Bt9*.

As previously mentioned in chapter 3 “Material and Methods”, five lines were inoculated with a different isolate. The spores for this isolate were collected from spikes of the cultivar “Tilliko”, hence the name “Tilliko-isolate”. In these five lines, inoculation with the Tilliko-isolate caused 13.67 % higher mean common bunt incidences than inoculation with the standard isolate. This leads to the assumption that different isolates have different levels of aggressiveness, hence different common bunt races cause different severities of disease incidence. Therefore, it is crucial to develop lines harboring diverse sources of resistance, such as multiple resistance genes, for example *Bt9* and *Bt11*. Such lines can be expected to provide more broad and durable resistance compared to those possessing only a single resistance gene.

IFA Tulln is currently conducting experiments with different isolates to test for their aggressiveness towards their most important breeding lines (including some lines from this master thesis) as well as towards differential lines. Furthermore, they test whether their currently well selected lines are resistant to isolates other than their established ones. In the future, hopefully projects like those at IFA – or more global ones like LIVESEED or ECOBREED – will achieve to develop lines and cultivars with diverse sources of resistance towards common bunt to ban this disease successfully and sustainably from organic agriculture.

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7 Appendix

7.1 DNA-Extraction on a 96-well plate with 1.2ml-8-stripes

Following the procedure described by Eric with slight adjustments.

Harvest young leaves.

Dry leaves: Dry them either at a temperature of ca 35°C (one to two days, according to leaf amount) or lyophilize tissue. When leaves are dried at 35°C, do not freeze leaves before drying.

NOTE: Leaf samples may be frozen and stored at -80°C until ready to be lyophilized. If leaves are frozen before drying you can only lyophilize them (72 hours). Frozen plant material must not thaw before lyophilizing. Make sure the lyophilizer is down to temperature (the chamber is ≤ -50°C) and pulling a good vacuum (≤ 10 microns Hg) before loading samples. Do not overload lyophilizer: make sure the vacuum is always ≤ 100 microns and condenser temperature is ≤ -50°C. Samples should be dry in 72 hours. Typically, fresh weight ≈ 10X dry weight. Dried leaf samples may be stored in sealed plastic bags at room temperature for a few days or at -20°C for several years.

1) **Prepare 1 set of 2 ml tubes.** Label tubes properly with a waterproof pen on cap and the side walls (the water bath may vanish your writing). Do not label with stickers, they will get loose in the water bath, and those which still stick to the tube seriously interfere with centrifuging. Write onto the cap of the tube the subsequent position number the samples will have in the 96 plate (1A, 1B, 1C, …, 12A, 12B, …, 12H) and on the walls the genotype number.

2) **Fill each tube with 5-7 small glass beads.**

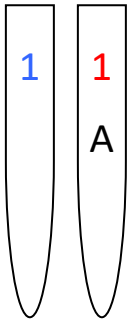
3) **Cut leaf-material into each tube, avoid contamination.** Bring dried leaves into 2 ml tubes (up to 50 µg), cut leaves when you fill the tubes. A small glass funnel makes filling easier. When you put your samples into an exicator overnight they are perfectly dry for grinding in the Retsch-mill. If leaves are not fully dried grinding will be poor. But the finer the powder the better the amount of extracted DNA!

4) Prepare 2 sets of 1.2 ml 8-stripe-tubes and 3 sets of caps and label them:

Labelling of caps (8-stripes):

	1	2	3	4	5	6	7	8	9	10	11	12
A	□	□	□	□	□	□	□	□	□	□	□	□
B		□	□	□	□	□		□	□	□	□	□
C			□	□	□	□			□	□	□	□
D				□	□	□				□	□	□
E					□	□					□	□
F						□						□
G												
H												

Labelling of tubes (8-stripes, side view):



row number top (A) / row number bottom (H)

plate name

5) Grind leaves in Retsch-mill for 10 minutes (change orientation of plates after 5 minutes). Ensure that both arms of the mill are encumbered equally. Alternatively use the shaker in BP/E/17 for ~20 minutes.

Leaf powder can be stored tightly capped in a cool place for several weeks or at -20°C (samples are stable for several years), or DNA extraction can begin immediately in the same tubes.

6) **Prepare CTAB-Buffer[1]: per sample 1 ml to 1.2 ml**

Stock	**final**	**5 ml**	**25 ml**	**50 ml**
dH_20		3.25 ml	16.25 ml	32.5 ml
1M Tris-7.5 (pH)	100 mM	0.5 ml	2.5 ml	5 ml
5M NaCl	700 mM	0.7 ml	3.5 ml	7 ml
0.5 M EDTA-8.0 (pH)	50mM	0.5 ml	2.5 ml	5 ml
CTAB[2]	1%	0.05 g	0.25 g	0.5 g
14 M BME[3]	140 mM	0.05 ml	0.25 ml	0.5 ml

[1] **use freshly made**; warm buffer to 60-65°C before adding the CTAB and BME.

[2] CTAB = mixed alkyltrimethyl-ammonium bromide (Sigma M-7635). **Do not breathe in!**

[3] add BME (β-mercaptoethanol) just prior to use, **under a fume hood**.

7) **Keep tubes at an appropriate distance** when opening them, open tubes carefully, so that leaf powder does not scatter and contaminate nearby tubes.

8) **Add 1000 µl - 1200 µl of CTAB-buffer to each tube under the fume hood.** If leaf powder sticks to the bottom you can stir it with a toothpick or a yellow tip, powder must get in contact with CTAB. Best is, to place the tubes into the heating block during this step.

   Close tubes tightly! Put tubes into racks.

9) **Shake by inversion and place the rack (with tubes) into a water bath (with gentle rocking) at 65°C for 60-90 minutes.**
   Use a rubber mat for fixing the racks in the metal trays and make sure that all caps and tube bottoms are covered by the mats. Close trays tightly.

10) **Let tubes cool down to room temperature.**

11) **Add 500 µl - 600 µl chloroform:isoamylalcohol (24:1) to each tube under the fume hood**, using Handystep for pipetting.

12) **Close tubes and put them into a rack, shake by gentle inversion for 10 minutes.**
All centrifugation-steps are done at room temperature!

13) **Centrifuge for 10 minutes, high speed.**

14) **Arrange tubes according to their alignment in the 96 plate, pipette off 700 (when 1000 µl CTAB) or 800 µl (when 1200 µl CTAB) of the top aqueous phase into 1.2 ml 8-stripe-tubes (properly labelled, see step 4).** Add 300 µl chloroform:isoamylalcohol (24:1), using the Handystep for pipetting, cover tubes, put plates into the plate-fasteners invented by Matthias, close screws tightly, shake by gentle inversion for 5 to 10 minutes.

The second chloroform cleaning step can be skipped, but by doing so pipette off less liquid (500 to 600 µl) to avoid any contamination between the two liquid phases. Transfer liquid into tubes containing RNase and continue with step 17.

15) **Centrifuge for 10 minutes, 3800 rcf,** Sigma 4K15 BP/E/21.

16) **Pipette off 300 µl (top aqueous layer) into a new tube containing 10 µl RNase A (1 mg/ml) and add new caps, incubate at room temperature for 30 minutes.**
Use a Brand Transferpette 8-chanel pipette for 30-300 µl by pipetting 300 µl, low speed, take the 8-stripe tubes one for one out of the plate for pipetting and make sure not to get any liquid of the bottom layer into the pipette tips.

17) **Mix by gentle inversion, incubate at room-temperature for 30 minutes.**
using the plate-fasteners

18) **Add 300 µl of isopropyl alcohol, mix well by gentle inversion.**
using the Brand-Handystep for pipetting

19) **Centrifuge 8 minutes at low rpm**
using the Sigma 4K15 BP/E/21 at ~600 rcf

20) **Pour off liquid (DNA-pellet must stick to the bottom of the tube).**
If the pellet does not stick to the bottom in some tubes, pipette off the liquid carefully.

21) **Add 100 µl of Wash 1 and mix gently for 5 minutes.**
using a Brand Transferpette 8-chanel pipette for 30-300 µl, pellets should be loose within the wash now

22) **Centrifuge 8 minutes at low rpm**
using the Sigma 4K15 BP/E/21 at ~600 rcf

23) **Pour off liquid (DNA-pellet must stick to the bottom of the tube).**
If the pellet does not stick to the bottom in some tubes, pipette off the liquid carefully.

24) **Add 100 µl of Wash 2 and mix gently for 5 minutes**
using a Brand Transferpette 8-chanel pipette for 30-300 µl, pellets should be loose within the wash now

25) **Centrifuge 8 minutes at low rpm**
using the Sigma 4K15 BP/E/21 at ~600 rcf

26) **Pour off liquid (DNA-pellet must stick to the bottom of the tube).**
If the pellet does not stick to the bottom in some tubes, pipette off the liquid carefully.

27) **Let dry overnight.**
Place racks in the right direction and put a tissue atop the open tubes. Make sure that the pellet is located on the bottom of the tubes and not up on the wall so that it is assured that it will dissolve in the buffer added the next day. The pellet cannot be seen any more after drying, it becomes transparent.

28) **Dissolve DNA-pellet in 100 µl in 0.1 or 0.05 x TE buffer**

Mix for at least half an hour at 65°C on a heated rocking device.
Then store the plates at 4°C in the fridge or continue with further analyses like photometric measurement of DNA-concentration and appropriate dilution.

7.2 Common bunt incidence of lines with or without awns

This boxplot shows common bunt incidence levels of lines of mapping population PI166910*SP with and without awns.

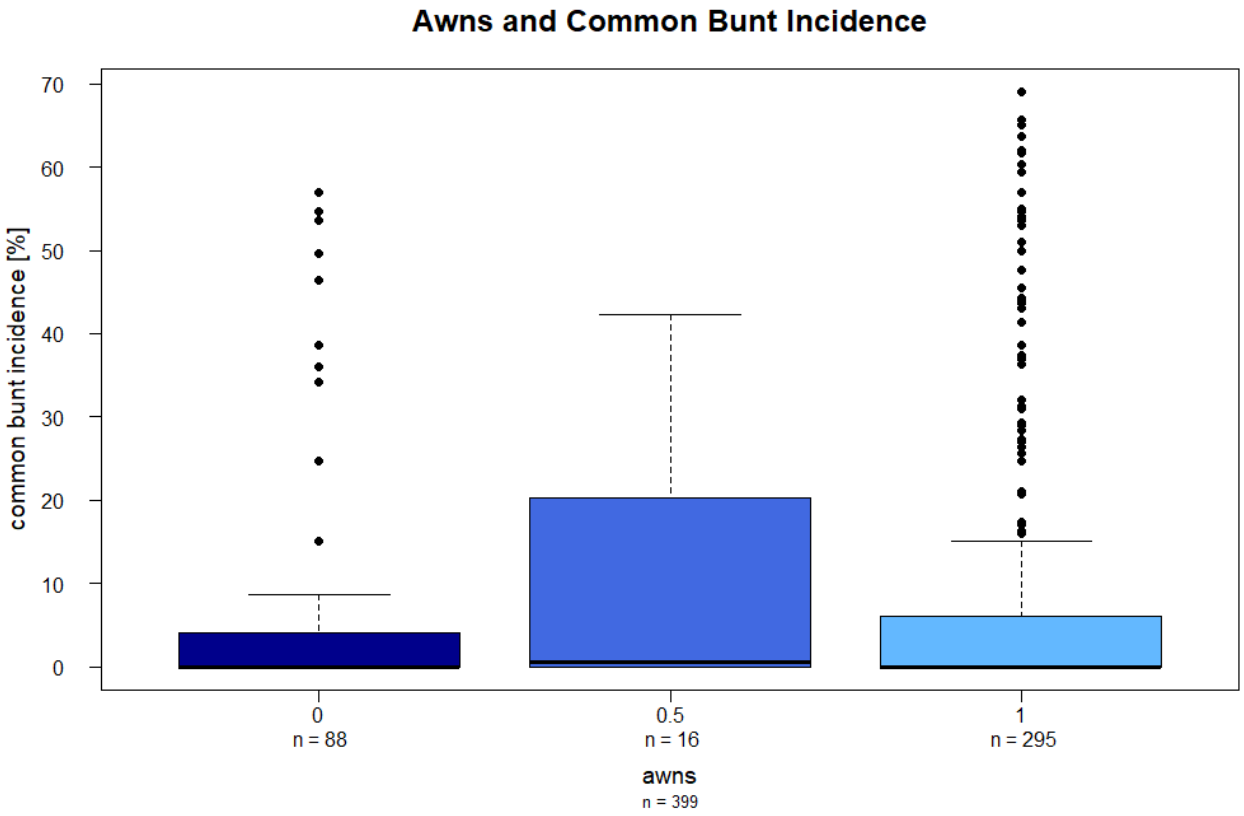


Figure 13. *Boxplot showing BLUEs of mapping population PI166910*SP for common bunt incidence levels of lines with (1), without (0) awns or with a mixed type (0.5), respectively.*

7.3 R-Code for statistical analysis

The following subsection shows excerpts of the R-code used for statistical analysis.

7.3.1 BLUEs

```
#initiate data frame:
blues <- NULL
#start loop – for c(5,6,7,8,9) insert columns with traits
for (i in c(5,6,7,8,9)) {
        #create model
        model.temp <- breedR::remlf90(fixed = Iris_data[,i] ~ -1 +
        Linie, random = ~ Wh, method = "ai", data = Iris_data)
        #extract BLUEs from the results of the model
        frame.temp <- as.data.frame(model.temp$fixed$Linie)
        #fill in data frame
        blues <- cbind(blues, frame.temp$value)
        blues <- as.data.frame(blues)
        #add column for names of genotypes
        blues$GEN <- rownames(frame.temp)
        #end of loop
}
#re-sort columns so that "GEN" is the first
blues <- blues[,c(2,1,3:ncol(blues))]
#re-name columns
colnames(blues) <- c("GEN", "DOH", "CBI", "Awns", "PH", "Rust")
```

7.3.2 ANOVA

```
#initialize data frame
anova <- data.frame(trait = character(),
                    Vg = numeric(),
                    Vrep = numeric(),
                    Ve = numeric())
for (i in c(5,6,7,8,9)) {
        #initialize temporary data frame
        frame <- NULL
        #create model
        model.anova <- breedR::remlf90(fixed = Iris_data[,i] ~ 1,
        random = ~ Linie + Wh , method = "ai", data = Iris_data)
        #extract variance components from the model
```

```
        Vg <- model.anova$var[1]
        Vrep <- model.anova$var[2]
        Ve <- model.anova$var[3]
        #transfer variance components to the data frame
        frame <- data.frame(trait = colnames(Iris_data)[i],
                            Vg = Vg,
                            Vrep = Vrep,
                            Ve = Ve)
        #connect temporary data frame with anova-dataframe
        anova <- rbind(anova, frame)
        #end of the loop
}
```

7.3.3 Repeatability

```
#h2=Vg/(Vg+(Ve/r))
#ve = error variance
#vg = genotypic variance
#vrep = variance of replication
#exemplary for CBI:
#extract variance components from ANOVA
Vg_CBI <- anova[2,2]
Ve_CBI <- anova[2,4]
r <- 2
#calculate repeatability
h2_CBI <- Vg_CBI/(Vg_CBI+(Ve_CBI/r))
```

7.3.4 Correlations for each combination of traits

```
#exemplary for CBI and DOH:
cor.test(blues$CBI, blues$DOH)
```

7.3.5 Linear models

```
#linear model using one marker as predictor for common bunt incidence
model_Iris_K140 <- lm(Iris_U10_U11$buntmean ~ ris_U10_U11$K140_allele)
summary(model_Iris_K140)
```

7.3.6 Wilcoxon rank sum test

```
#group levels of allele calls as factors
Iris_K140$K140_allele <- as.factor(Iris_K140$K140_allele)
str(Iris_K140)
#grouping factor must have exactly two levels - adjust allele calls
Iris_Wilcox140 <- Iris_K140[Iris_K140$K140_allele == "Allele 1" |
   Iris_K140$K140_allele == "Allele 2", c(1,2,6)]
Iris_Wilcox140 <- droplevels(Iris_Wilcox140)
#perform Wilcoxon rank sum test
wilcox.test(Iris_Wilcox140$buntmean ~ Iris_Wilcox140$K140_allele,
   alternative = "two.sided", exact = FALSE)
```

7.4 Significance levels of variance components

This sub-section shows significance levels of variance components presented as p-values for mapping populations PI166910*SP (Table 23) and 702-1102C*SP (Table 24).

Table 23. *Significance levels of genetic variance and replication variance for mapping population PI166910*SP for common bunt incidence, plant height, date of heading, brown rust incidence and presence or absence of awns given as p-values (α = 0.5).*

	CBI [%]	**PH [cm]**	**DOH**	**BRI [%]**	**Awns**
$\sigma^2_{Genotype}$	<2e-16	5.29e-16	<2e-16	<2.2e-16	<2e-16
$\sigma^2_{Replication}$	0.0341	<2.2e-16	0.069	<2.2e-16	1

Table 24. *Significance levels of genetic variance and replication variance for mapping population 702-1102C*SP for common bunt incidence, plant height, date of heading, brown rust incidence and presence or absence of awns given as p-values (α = 0.5).*

	CBI [%]	**PH [cm]**	**DOH**	**BRI [%]**	**Awns**
$\sigma^2_{Genotype}$	<2e-16	0.003	1.127e-11	1.054e-7	-
$\sigma^2_{Replication}$	0.1677	0.026	0.071	<2.2e-16	-

7.5 Climate data

This chapter shows precipitation and temperature during the growing period 2018/19 in Tulln as well as long term data between 1981 and 2010 in Langenlebarn.

7.5.1 Precipitation

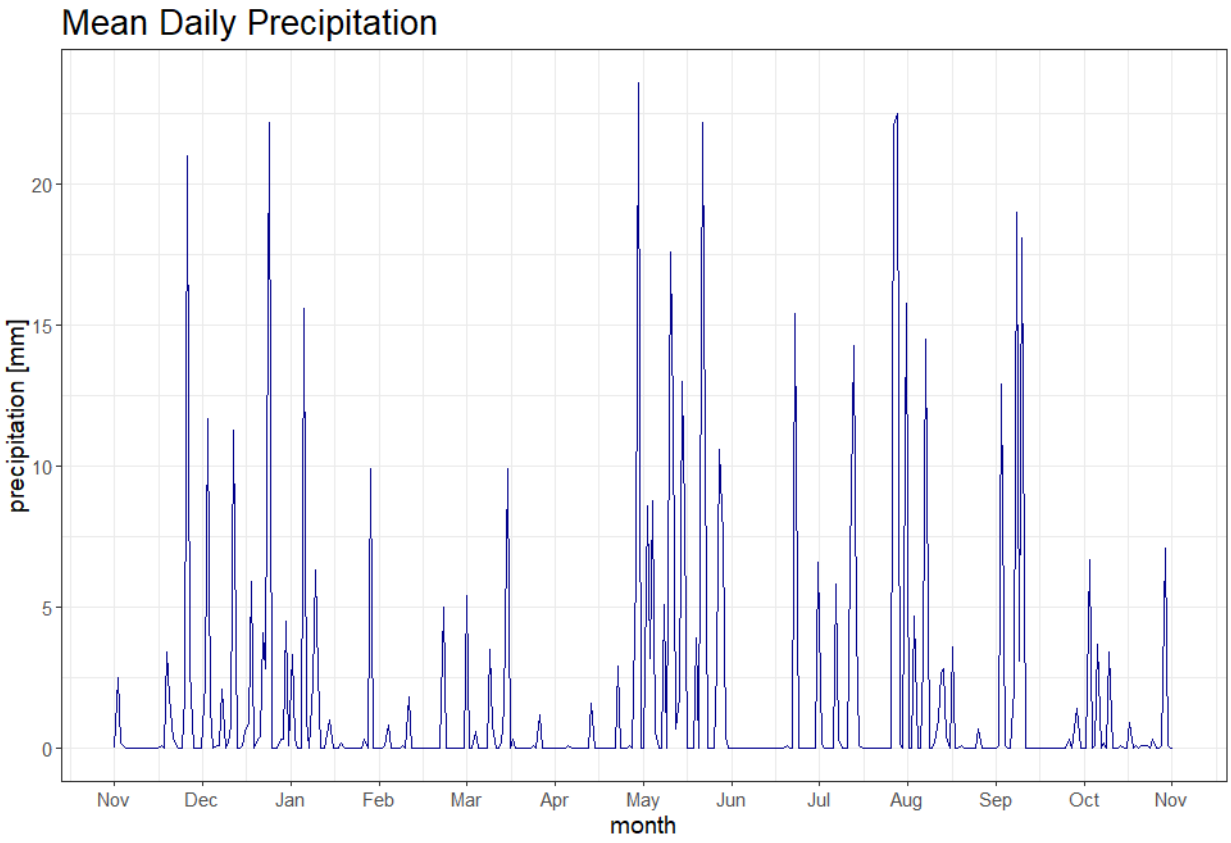


Figure 14. *Mean daily precipitation in Tulln, Austria for the growing period 2018/19 (Nov. 1st, 2018 to Oct. 31st, 2019).*

7.5.2 Temperature

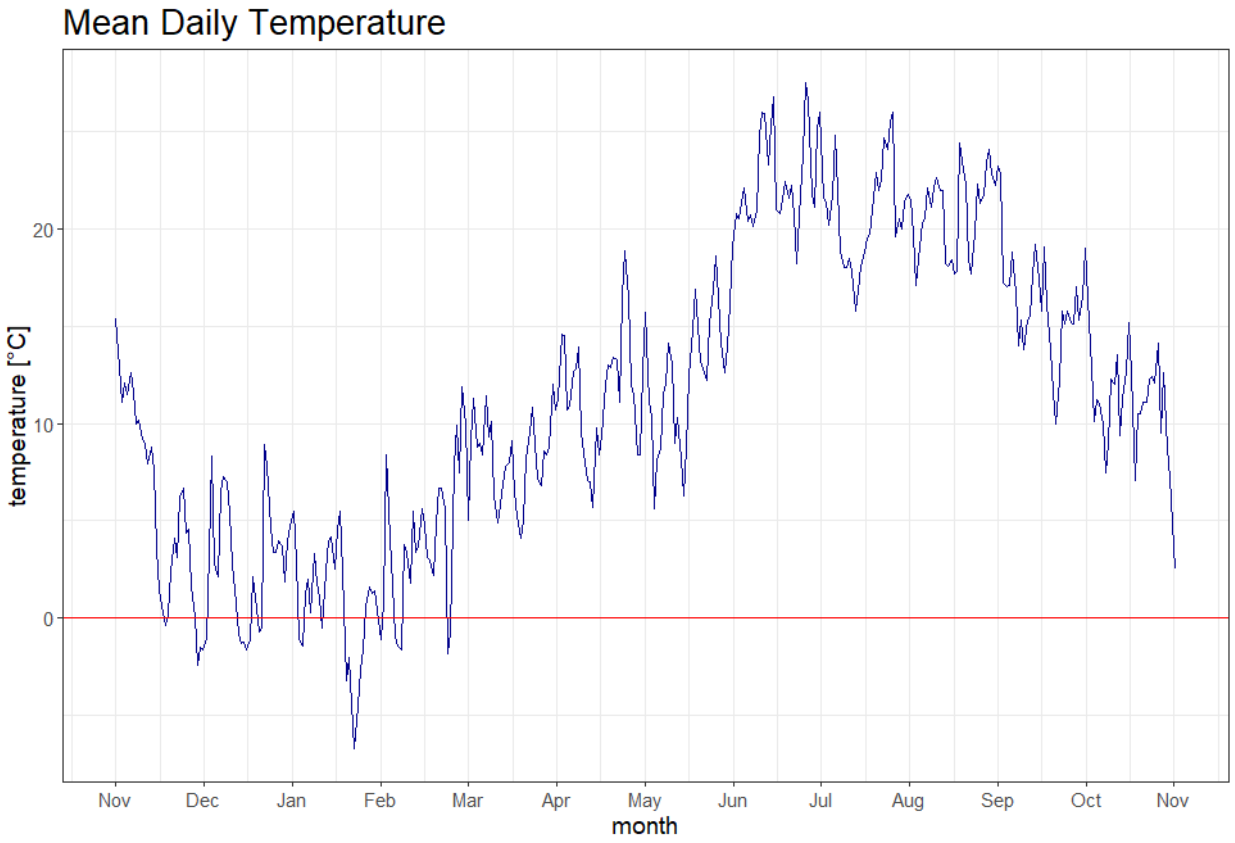


Figure 15. *Mean daily temperature in Tulln, Austria in the growing period 2018/19 (Nov. 1st, 2018 to Oct. 31st, 2019).*

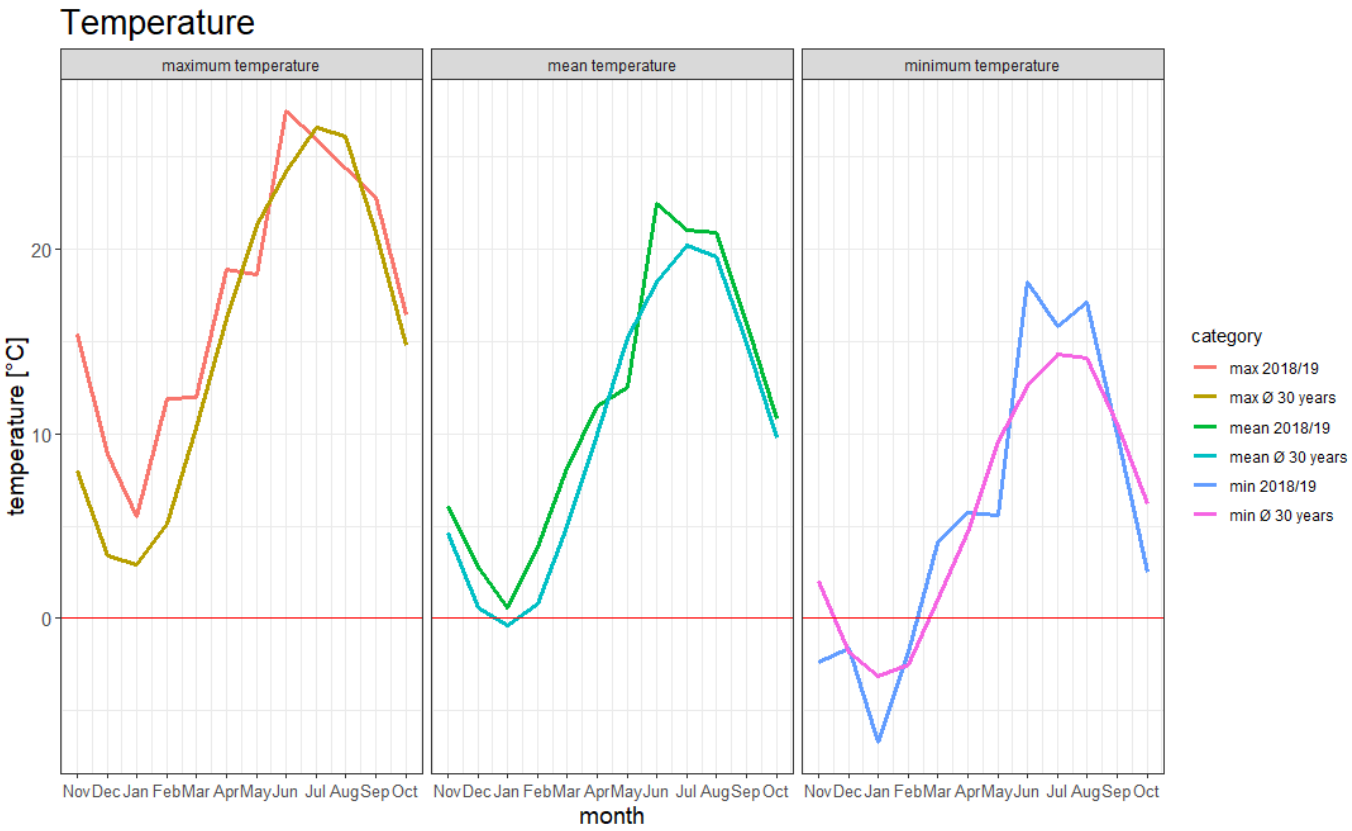


Figure 16. *Maximum temperatures in Tulln, Austria for each month of the growing period 2018/19 (Nov. 1st, 2018 to Oct. 31st, 2019) compared to the maximum monthly temperatures in Langenlebarn, Austria between 1981 and 2010 (left). Mean temperatures in Tulln, Austria for each month of the growing period 2018/19 (Nov. 1st, 2018 to Oct. 31st, 2019) compared to the mean monthly temperatures in Langenlebarn, Austria between 1981 and 2010 (middle). Minimum temperatures in Tulln, Austria for each month of the growing period 2018/19 (Nov. 1st, 2018 to Oct. 31st, 2019) compared to the minimum monthly temperatures in Langenlebarn, Austria between 1981 and 2010 (right).*