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Characterising resilience and resource-use efficiency traits from Scots Bere and additional landraces for development of stress tolerant barley

Jonathan Cope

BSc (Hons) from the University of St Andrews
MSc from the University of East Anglia

Supervised by:
Adrian C Newton (James Hutton Institute)
Timothy S George (James Hutton Institute)
Gareth Norton (University of Aberdeen)

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Abstract

With a growing population, it is important to increase crop yield. However, there is a low priority in breeding for increased tolerance to low input or marginal environments. Potential sources of viable resilience and resource-use efficiency traits are landraces local to areas of marginal land, such as the Scots Bere from the Highlands and Islands of Scotland. Bere barley is a deeply historically rooted landrace of barley that has been grown on predominately marginal land for the last half millennia. The landrace yields well in these conditions. The project aim was to assess and genetically characterise traits associated with enhanced resistance/tolerance, and to identify contributing genomic regions.

The JHI spring barley collection, consisting of a number of Bere lines, was screened for biotic stress resistance to *Rhynchosporium commune* and abiotic stress resistance to the conditions of manganese (Mn) deficiency and salt stress. Additionally, the interaction of these stresses was assessed. The results identify a number of Bere lines that show an increased resistance/ tolerance to each of the three stresses, compared to elite cultivars. The Bere population, as a whole, showed an inherent enhanced Mn-use efficiency, correlating to increased accumulation of Mn in the shoots. These results suggest that Bere landraces have unique abilities to cope with stress. Interaction studies revealed complex line-specific interactions, along with an overall adverse effect of salt on rhynchosporium symptoms.

Several genomic regions for Mn-use efficiency, salt tolerance, and rhynchosporium resistance traits, originating from the Bere lines, were identified, along with potential candidate genes. Further examination and validation of these regions should be undertaken for future breeding for marginal lands. By introgression into elite cultivar backgrounds, they may contribute biotic and abiotic stress-tolerance genes. This could create novel cultivars to efficiently and resiliently yield under low input and marginal environments.

General Introduction

Barley Crop

The diploid species *Hordeum vulgare* is the most widely spread *Hordeum sp.*, comprised of all genotypes of cultivated barley and its wild ancestor which is still abundant in Southwest Asia. Originally *H. vulgare* was split into three separate species; the wild form *H. spontaneum*, the cultivated two-row form *H. distichon*/*distichum*, and the cultivated six-row form *H. hexastichon*/*hexastichum*. However, based on several factors (including successful interbreeding to produce fertile offspring), these *Hordeum spp* are now classified into two different subspecies within the same species; *H. vulgare ssp. spontaneum*, and *H. vulgare ssp. vulgare*. The cultivated form, *H. vulgare ssp. vulgare*, further separates into two taxonomical varieties to distinguish between two-row and six-row; *var. distichon*, and *var. hexastichon*, respectively (von Bothmer *et al.,* 2003; Khodayari *et al.,* 2012; Zohary *et al.,* 2012).

Whilst the location of origin for the *Hordeum* genus is currently attributed to a large area, *H. vulgare ssp. spontaneum* is widely regarded to originate in the fertile crescent, an area of Southwest Asia that stretches from the Persian Gulf to Northern Egypt which is widely regarded as the birthplace of agriculture (Mark, 2009; Dai *et al.,* 2012). Recent archaeological evidence from a hunter gatherer camp site on the shore of the Sea of Galilee, Israel, points to the use of wild barley as a human food source as far back as 21,000 BCE; it was also shown that humans had developed tools to process the grains (Nadel *et al.,* 2012; Snir *et al.,* 2015). The Fertile Crescent is home to multiple wild species, including wild wheat (Brown *et al.,* 2009). It was in this area that the origins of cereal crop domestication began with the domestication of barley, along with early wheat, creating *H. vulgare ssp. vulgare*. This domestication event is thought to have occurred approximately 8,000 BCE from evidence of domesticated barley remains in archaeological sites from that period. The presence of early wheat was also found but in smaller numbers, indicating that Barley was more common in Neolithic agriculture (Newman and Newman, 2006; Dai *et al.,* 2012).

General Introduction

Archaeological evidence has shown that both two-row and six-row cultivated barley have been grown in large quantities since 5,000-6,000 BCE in the Fertile Crescent (Renfrew, 1969; Newman and Newman, 2006; Cocks, 2013). It is widely accepted that the domestication of barley establishes it as one of the founder crops in the development of agriculture, and the principle crop in the spread of agriculture from South-west Asia outwards. Originally, in the Mesopotamian region, barley was established as the main crop over wheat, partially due to the ability of barley to adapt to more extreme regions (von Bothmer *et al.,* 2003; von Bothmer and Komatsuda, 2010; Zohary *et al.,* 2012). This period is known as the Neolithic Revolution, where mankind transitioned from nomadism to sedentary communities. During this era of growing cultivated barley on a large scale the production of fermented barley, beer, was developed. The earliest evidence of a fermented beer-like beverage has been found in China 8,000 BCE (McGovern *et al.,* 2004; Pires and Brányik, 2015). However, it is thought that beer has been made with barley (along with other grains) for 6,000-11,500 years (Hornsey, 2003; Haaland, 2007; Stordeur and Willcox, 2009; Sicard and Legras, 2011; Hayden *et al.,* 2012). It has also been proposed that the act of discovering the process of fermentation led to the increased demand of cereal crops like barley, causing the Neolithic Revolution and thus creating primary civilisations (Braidwood *et al.,* 1953; Katz and Maytag, 1991; Joffe, 1998; Damerow, 2012).

Currently, worldwide production of barley ranks it as the 12th most popular crop in terms of tonnage produced. In the UK barley is the 2nd most highly produced crop with a production of over 6.5 million tonnes in 2016, worth a net production value of over $350 million making it the UK's 4th most valuable crop (FAOSTAT). Of the barley produced worldwide approximately 5-7% is saved as seed for farming or research, the remainder has three main uses; feed (60-70%), malting (20-33%), and food for human consumption (2-5%). However, this is greatly variable between countries as some countries with extreme climates and marginal agricultural environments, such as Ethiopia and Morocco, use barley as a principal food source (Ceccarelli *et al.,* 2007; Baik and Ullrich, 2008; Newman and Newman, 2008). In wealthier countries malting barley is heavily produced due to the higher value, in the UK 35% of all barley is used for malting (NFU and BBPA, 2013).

Genetic Diversity

Harlan and de Wet (1971) proposed a method of gene pool classification which assigns taxa related to a crop to a primary, secondary, or tertiary gene pool. The primary gene pool is defined by the species of the crop, containing all subspecies within, making crossing easy and with the majority of hybrids produced fertile. The secondary gene pool contains all species that will cross with the crop producing some offspring with fertility, but that may be difficult to achieve. The tertiary gene pool includes closely related species that will cross with the crop, but produce no fertile offspring so that gene transfer from this group cannot be achieved through conventional breeding. von Bothmer *et al.* (2003) outline what this classification is for the barley crop (Figure 1). The primary gene pool includes all commercial cultivars, breeding lines, landraces, and wild barley (*H. vulgare ssp. spontaneum*). The secondary gene pool includes *H. bulbosum*, the other *Hordeum sp.* that contains the H genome similar to barley. *H. bulbosum* is used to create hybrid double haploids with *H. vulgare* in the "*Hordeum bulbosum* method" (Devaux, 2003). The Tertiary gene pool includes all the other *Hordeum* spp.

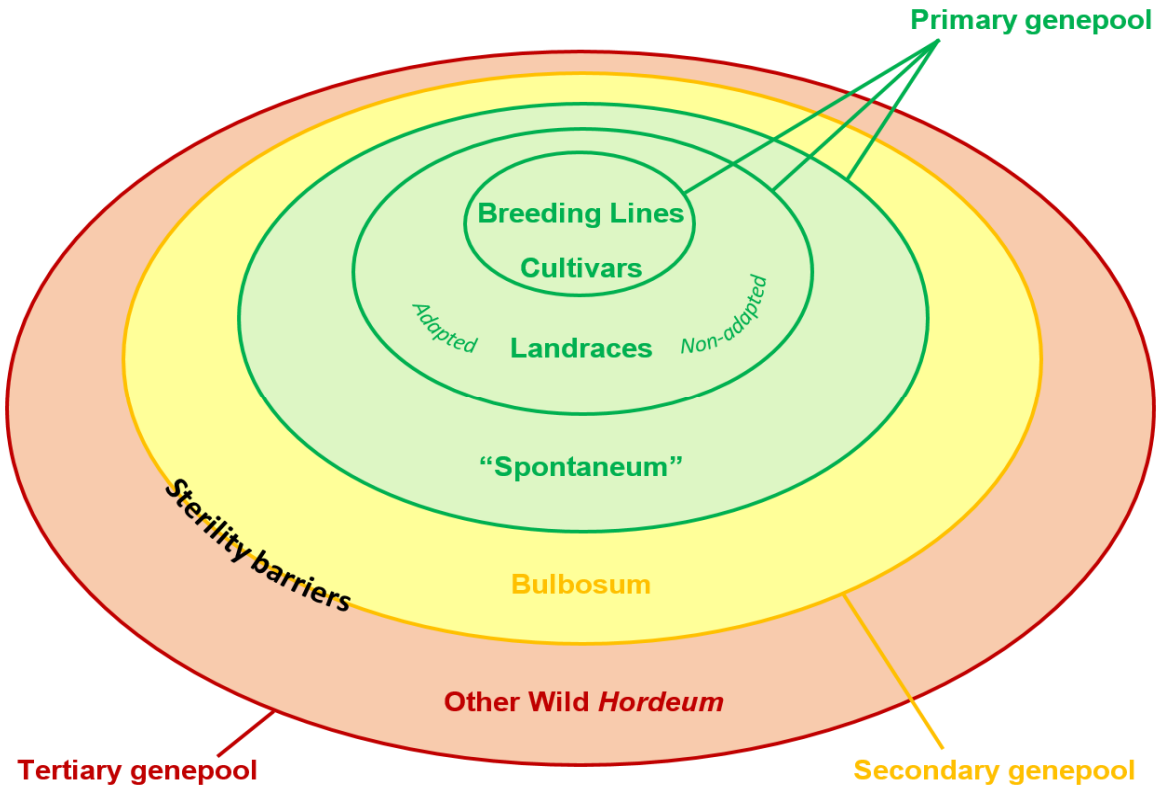


Figure 1) Distribution of the barley breeding gene pools in the method outlined by Harlan and de Wet (1971). Figure adapted from von Bothmer *et al.* (2003).

1.1.1.1. Landraces

Landraces are distinct but heterogeneous populations that are maintained through continuous multiplication within a specific regional environment, which include climate and soil conditions along with cultivation techniques. These practices subject the cultivars to both natural and artificial selection, and so they are often more locally adapted than other cultivars. Barley landraces were established in all areas where barley was grown and, due to the minimal transfer and the practice of farm saved seed, the local populations diversified (Poets *et al.,* 2015). The use of these landraces was universal until the 19th century where, in the UK, 'improved' seed cultivars were chosen in favour. These were bred using landraces from other regions to develop elite cultivars such as Archer, Spratt, and Chevalier. The development of modern plant breeding in the 20th century saw the rise of elite cultivars that were selected, usually from a single genotype, for improvements in traits such as yield, seed quality, and biotic resistance. These new elite cultivars replaced many of the landraces in Europe during the 1920's leaving only landraces in poor agricultural environments or remote areas, thus much genotypic material was lost (Fischbeck*,* 2003; Leino and Hagenblad*,* 2010; Bellucci *et al.,* 2013).

Recently there has been an effort to preserve the genotypic diversity held in the population of landraces. In recent years efforts have been made to calculate the total number of accessions held between all the institutes around the world, these estimations are between 370,796 – 466,531 accessions in total, making barley the third most populace *ex situ* genebank collection, after wheat and rice (van Hintum and Menting*,* 2003; FAO*,* 2010). However, whilst this gene bank saves genetic data that could otherwise be lost, it is not completely effective. Parzies *et al.* (2000) have shown that, due to the necessity of accession rejuvenation approximately every 5 years causing genetic drift, there is a very significant decline in genetic diversity with time.

The variation between landraces shows a distinct geographic structure, with four main groups identified: Coastal Mediterranean, Central European, East African, and Asian (Poets *et al.,* 2015). In areas where there is a concentrated representation of landraces, the variation between them has shown localised geographic structures, as shown in the landrace material from the Himalayas (Pandey *et al.,* 2006), Spain (Yahiaoui *et al.,* 2007), and Sweden (Leino and Hagenblad*,* 2010). A

study by Bellucci *et al.* (2013) demonstrated that the landrace populations are variable populations containing a large number of different genotypes.

The diversity of landraces is due to their local adaptation via natural and artificial selection, with likely evolutionary contributions from *H. vulgare* ssp. *spontaneum*. They are currently often confined to marginal lands, where the elite cultivars cannot outperform them and thus it is not economically favourable to replace them (Abera, 2009; Yahiaoui *et al.,* 2014). It is due to this diversity of genetic material that landraces offer a substantial genetic potential for breeders. The germplasm offers a potential for increased nutrient uptake and efficiency traits, improved nutrition (particularly antioxidants), a bank of resistance mechanism to both biotic and abiotic stress, and characters useful for low input agriculture. Thus this material is being favoured for breeding to increase yield in the harsh agro-ecological and climatic conditions of marginal land (Newton *et al.,* 2010).

A common biotic resistance trait that developed in barley landraces, and can be used in breeding programs, is resistance to powdery mildew. The most highly used resistance gene for powdery mildew, and one of the most successful durable resistances to any important crop pathogen, is *mlo* that was identified in Ethiopian landrace material collected in the 1930's (Jørgensen, 1992). Newer resistance genes to this fungal pathogen have also been identified in Spanish (Silvar *et al.,* 2011), Libyan (Czembor and Czembor, 2002), Jordanian (Abdel-Ghani *et al.,* 2008), Moroccan (Czembor, 2000b), Egyptian (Czembor, 2000a), Tibetan (Zeng *et al.,* 2014), Czech, and Slovakian (Dreiseitl and Jørgensen, 2000) landraces. Resistance genes to multiple other pathogens have also been identified, including: 1) Scald pathogen (*Rhynchosporium commune*) – found in landraces from Ethiopia (Yitbarek *et al.,* 1998; Bjørnstad *et al.,* 2004), Syria, and Jordan (van Leur *et al.,* 1989) and particularly high levels of resistance found in lines derived from Spanish landraces (Hofmann *et al.,* 2013). 2) Leaf Rust pathogen (*Puccinia hordei*) – found in landraces from Ethiopia (Alemayehu and Parlevliet, 1996), former Yugoslavia (König *et al.,* 2012), and the Southern Mediterranean region (Czembor and Czembor, 2007). 3) Fusarium diseases – with resistance to Fusarium Crown Rot found in a landrace from Japan (Chen *et al.,* 2013), and Fusarium Head Blight Resistance identified in landraces from Ethiopia and Eritrea (Mamo and Steffenson, 2015). 4) Stem Rust pathogen (*Puccinia graminis* f.sp.

tritici) – much of the resistance to this pathogen was overcome by a Ugandan lineage, Ug99 (Race TTKSK), that has broad spectrum resistance; recently landraces from Switzerland have been identified with high levels of resistance to this Stem Rust race that has become a serious threat to barley, and wheat, production (Mamo *et al.,* 2015).

Due to the continuous growth of landraces on marginal soils, they can potentially be a valuable source of genetic material for tolerances against abiotic stresses. One of the most common abiotic constraints is drought, affecting large regions of low rainfall areas that depend on rain-fed water application, in both more and less economically developed countries. Landraces have been a large source of drought tolerance in arid regions such as Ethiopia (Abera*,* 2009), Namibia (Ben Naceur *et al.,* 2012), and particularly in the region of the fertile crescent such as Syria (Grando *et al.,* 2001), Iran (Pour Aboughadareh *et al.,* 2013), and Jordan (Haddadin*,* 2015). A study showed that in arid and semi-arid environments, adaptation to excessive irradiance was an important factor in drought tolerance, this study indicated that a Syrian landrace has this adaption (Tardy *et al.,* 1998). Prolonged drought stress events in otherwise water adequate environments is an alternative, but related, water deficient stress; landraces from the Mediterranean region have shown a tolerance that could be utilised (Comadran *et al.,* 2007). Like drought, salt stress is an osmotic stress, and thus likely has overlapping mechanisms of tolerance. Similarly landraces that express tolerance have been identified in populations from Morocco (El Madidi *et al.,* 2004), Oman (Jaradat *et al.,* 2004), and Syria (Kalaji *et al.,* 2011), along with an Algerian landrace that has also been shown to have boron tolerance and cereal cyst nematode resistance (Karakousis *et al.,* 2003; Hayes and Reid*,* 2004; Widodo *et al.,* 2009). In other agricultural areas, often in the most northerly/southerly regions or at high altitudes, frost is a major abiotic stress. Major frost tolerance genes from landraces found in the Turkish highland regions have been widely used in winter barley breeding programs (Akar *et al.,* 2009; Newton *et al.,* 2010). Other landraces that display frost tolerance have been found in the Ethiopian highlands (Eticha *et al.,* 2010; Shewayrga and Sopade*,* 2011; Fetene *et al.,* 2012).

1.1.1.2. Scots Bere

The Scottish landrace ‘Bere’ has been grown on predominately marginal land for, at least, the last half millennia, and currently is grown on the highlands and islands of Scotland. It is thought that this landrace was first introduced to the northern areas of Scotland around the 8th century by Danish and Norse invaders, with the name ‘Bere’ coming from the Old Norse word ‘Bygg’ meaning barley (Jarman*,* 1996). Bere barley may also have been one of the first barley varieties introduced to the Pacific Northwest during the European colonisation of North America (Scheuerman and McGregor*,* 2013). Bere is of the *hexastichon* variety, 6-row, and is a spring barley that is traditionally sown in late spring, with rapid growth allowing harvest in just 90 days. However, this later sowing is thought to be established due to work prioritisation and has continued as tradition. It has been shown that a significant yield advantage can be acquired by planting the Bere barley earlier, towards the end of April (Martin *et al.,* 2010). Historically, it was Scotland’s main barley crop and was used for all the barley uses of that time; including food, feed, and malting, with the straw being used for animal bedding and thatching (Martin *et al.,* 2009).

Current uses are much more limited with much of the Bere cultivation being replaced with higher yielding and shorter straw cultivars in much of the Highlands and Islands, with grass pastures for cattle grazing in Orkney (Martin *et al.,* 2008a), and recently in the Shetlands much of the remaining Bere barley was replaced by sheep farming (SASA*,* 2015). The cultivation of Bere barley in Orkney has been sustained due to the association with a traditional water mill, Barony Mill. This mill processes the grain to produce Bere meal, a type of flour similar to what would have been produced in historic times. This Bere meal is used around the island to make products such as bread, biscuits and the traditional Bere meal bannock, a type of traditional scone (Martin *et al.,* 2008b).

The main concerns of growing Bere barley, as noted by farmers interviewed in 2003, is lodging and the low yielding aspects. Yields for Bere barley are typically half that of commercial elite varieties grown in similar climatic conditions (in Orkney) with optimum applications, but Bere populations are showing limited yield response to applications of N, P, or K fertilisers (Martin *et al.,* 2008a). Yields for Bere populations were able to be increased by 47% one year by a combination of earlier planting

date, highlighted above, along with both fungicide and growth regulator (Martin *et al.,* 2009), suggesting the low yields of Bere barley may be improved slightly with altered agronomic practices. Emphasising the low yielding aspects of Bere outside its native location is an organic spring barley trials in Washington state undertaken by Jones and Lyon (2012). Grown with organic fertilizer applications, this trial showed the yield of the single Bere barley variety tested was the lowest yielding of all varieties tested, with less than one third of the highest performing variety (Brouwer *et al.,* 2015).

However, Bere, like other landraces and rare breed products, can sell for a premium due to a combination of rarity/novelty, promotion of local products and practices, heritage appeal, and generation of ecosystem services (ES) (Riu-Bosoms *et al.,* 2014; Villa *et al.,* 2006; Ovaska and Soini, 2016; Heinonen and Veteläinen, 2011). This value-adding trait, along with potential government subsidies to promote agrobiodiversity, makes the production of Bere barley economical; but in limited cases at present, with possible further cases if the production costs could be reduced. Examples of the commercial products that include specialist whiskies and beers made with Bere barley. This allowed the products to sell for a premium, negating the extra costs accrued due to the higher cost of the lower yielding grain, higher cost of malting smaller quantities of grain, and the need for a higher quantity of grain per bottle produced. The latter effect is due to the reduced sugar extraction from the Bere malt, caused by a larger protein content resulting in a reduction in available starch compared to elite malting varieties (Martin and Chang, 2008; Martin and Wishart, 2015) estimated to be a 15-20% lower yield of sugars in the wort (Martin *et al.,* 2008a). This premium, however, is limited to local markets due to the lack of historical and cultural ties to the crop in other regions (Mahon *et al.,* 2016).

Within the literature Bere barley is generally referred to as a single variety and is often shown to have different, sometimes conflicting, phenotypes. This diversity is due to the isolated nature of the islands where Bere is still grown. A study undertaken by Southworth (2007) using 29 microsatellite markers shows that there is significant genetic variance between the 3 island groups of the Shetland, Orkney, and Western Isles. It was suggested that this clustering is due to the lack of historical seed trade between the island groups, but also suggested that it could be due to adaptation to the differing environments between the islands. This diversity was compared with the diversity of 134 cultivars on

the national list, over 5 loci, and showed that the Bere lines have similar levels of diversity as found in the pool of elite cultivars. Whilst this diversity between island groups was high, over two-thirds of the total diversity was found within the island group clusters. The Western Isles displayed the greatest genetic diversity potentially due to similar geological constraints as between the island clusters.

As stated, this diversity manifests as differing phenotypic traits with regards to both biotic and abiotic stresses, potentially differing due to unique nutrient deficiencies and toxicities found in the different environments of the islands farming area. Some Bere lines have been shown to be tolerant to acidic soils (Wright *et al.,* 2002; Ellis*,* 2004), found to be regulated by a single gene on chromosome 4H and possibly indicating aluminium tolerance (Stølen and Andersen*,* 1978; Wright *et al.,* 2002). Local farmers also noted that Bere barley was able to grow well on the poorly drained peat soils found in the Shetlands, possibly due to the low pH tolerance (Mahon *et al.,* 2016). Work by George *et al.* (2014) and Schmidt *et al.* (2018) has shown that other Bere lines maintain optimal growth in alkaline soils, such as the Machair in the Western Isles, and the associated manganese deficiency. Therefore these, and potentially other Bere landraces, offer a promising source of Mn use efficiency genes that can be used in breeding. Southern Australian barley breeding programs, for example, have used a group of two RFLPs on the short arm of chromosome 4 (4HS), linked to a locus of manganese efficiency designated *Mel1*. The *Mel1* locus was found in a Mn-efficient variety called Amagi Nijo and assessed using bulk segregant analysis (Pallotta *et al.,* 2000; Pallotta *et al.,* 2003; Poulsen and Lance*,* 2010). With regard to biotic stresses, it has been observed that Bere barley has an increased susceptibility to powdery mildew (*Blumeria graminis* f.sp. *hordei*), and possibly other foliar diseases (Wright *et al.,* 2002) such as barley leaf strip (*Pyrenophora graminea*), when compared to other Scottish barley seed (Cockerell*,* 2002). However, in an interview in 2003 with farmers who grew Bere barley in Orkney, the susceptibility to powdery mildew was not noted by most as a concern (Martin *et al.,* 2009). Later interviews with farmers by Mahon *et al.* (2016) suggest that Bere are more resistant to other foliar diseases, in particular barley leaf scald (*Rhynchosporium commune*) AKA barley leaf blotch. This difference in reported and observed susceptibility could be due to differences within the Bere population.

Biotic Stresses in Barley

Biotic stress is defined as the stress caused by independent organisms or pseudo-organisms, including: a) macroscopic organisms such as insects, grazing animals, weeds, and competing crops, b) microscopic agents such as fungus, bacteria, nematodes, and protists/protozoa, and c) pseudo-organisms including viruses and sub-viral agents such as viroids (Agrios, 2005; Schumann and D'Arcy, 2006; Newton *et al.,* 2011a). Crop losses for the six major crops were compared by Oerke (2005) showing that weeds displayed the biggest potential for losses in all crops, but due to effective management the actual crop loss due to pathogenic microbes were bigger and/or the biggest for most crops (Table 1). These microscopic organisms along with viruses are the primary causal agents responsible for diseases in plants (Gimenez *et al.,* 2018), which is estimated to be responsible for the loss of at least 10% of food production globally (Strange and Scott, 2005) and a potential of 18.1% loss in wheat (Table 1), a comparable crop to barley. Of these plant diseases, fungal pathogens are prominent as causal agents of economically important diseases in cereal crops such as wheat (Figueroa *et al.,* 2018), rice (Gnanamanickam, 2009), and barley (Newton *et al.,* 2011a).

Table 1) Comparison of potential and actual global crop loss worldwide in six major crops, broken down by type of biotic stress, for the 2001-2003 period. Table adapted from Oerke (2005).

	Crop		Wheat	Rice	Maize	Potatoes	Soybeans	Cotton
	Attainable production [Mt]		785	933.1	890.8	517.7	244.8	78.5**
Crop losses* (%) due to:	Weeds	Potential	23.0 (18–29)	37.1 (34–47)	40.3 (37–44)	30.2 (29–33)	37.0 (35–40)	35.9 (35–39)
		Actual	7.7 (3–13)	10.2 (6–16)	10.5 (5–19)	8.3 (4–14)	7.5 (5–16)	8.6 (3–13)
	Animal Pests	Potential	8.7 (7–10)	24.7 (13–26)	15.9 (12–19)	15.3 (14–20)	10.7 (4–16)	36.8 (35–41)
		Actual	7.9 (5–10)	15.1 (7–18)	9.6 (6–19)	10.9 (7–13)	8.8 (3–16)	12.3 (5–22)
	Pathogens	Potential	15.6 (12–20)	13.5 (10–15)	9.4 (8–13)	21.2 (20–23)	11.0 (7–16)	8.5 (7–10)
		Actual	10.2 (5–14)	10.8 (7–16)	8.5 (4–14)	14.5 (7–24)	8.9 (3–16)	7.2 (5–13)
	Viruses	Potential	2.5 (2–3)	1.7 (1–2)	2.9 (2–6)	8.1 (7–10)	1.4 (0–2)	0.8 (0–2)
		Actual	2.4 (2–4)	1.4 (1–3)	2.7 (2–6)	6.6 (5–9)	1.2 (0–2)	0.7 (0–2)
	Total	Potential	49.8 (44–54)	77.0 (64–80)	68.5 (58–75)	74.9 (73–80)	60.0 (49–69)	82.0 (76–85)
		Actual	28.2 (14–40)	37.4 (22–51)	31.2 (18–58)	40.3 (24–59)	26.3 (11–49)	28.8 (12–48)

* Figures in parentheses indicate variation among 19 regions.

** Seedcotton.

Fungal pathogens are grouped into three broad categories depending on the state of the plant host tissue that the fungus feeds on. Biotrophs are fungi that feed on the living host tissue whilst necrotrophs kill and feed on the dead tissue, the third category are hemi-biotrophs that behave as both a biotroph and necrotroph depending on their environment and/or stage in their lifecycle (Glazebrook, 2005; Vleeshouwers and Oliver, 2014). Infection of plant material for most pathogenic fungi starts with adherence to the plant surface cells, growth of germ tubes, and then penetration into

the plant using various infection structures. After penetration, necrotrophic fungi tend to grow sub-cuticularly whilst releasing toxic proteins and metabolites that kill the cells around them, allowing the fungal hyphae to replace the cells. Biotroph hyphae can grow both inter- and intracellularly, the latter of which often encases its hyphae in the hosts plasma membrane and can grow dedicated feeding structures within the cells; some biotrophic fungi are exclusive intercellular colonizers. Within the biotrophic grouping there are obligate biotrophs that rely solely on the host for nutrients, and facultative biotrophs that use the host as an alternative source of nutrients. Hemibiotrphic fungi tend to have larger intracellular hyphae during their biotrophic phase, and then develop into thin hyphae for the necrotic phase (Lo Presti *et al.,* 2015).

Fungal diseases are particularly prevalent in monoculture farming techniques, commonly practiced, providing an increased selection pressure allowing pathogens to overcome resistances within the crop more easily (Oerke*,* 2005). Resistance can also be affected by abiotic stresses, either temporarily or permanently. For example, the temporary compromise of barley's resistance to powdery mildew, via the *mlo* resistance gene, caused by the rapid relief of drought (Newton and Young*,* 1996). Changes in climate will add additional problems with fungal pathogens as differing environments could change the pathogens behaviour and shift the patterns of infection so that diseases move into new regions with the susceptible host crops. Together these show a current and future threat of fungal pathogens and need to address them through means such as breeding programmes and agronomic practices (Newton *et al.,* 2011a; Chakraborty and Newton*,* 2011).

Plant innate immune system consists of two layers: PAMP-triggered immunity (PTI), and effector-triggered immunity (ETI). Pathogen-associated molecular patterns (PAMPs) are molecular patterns that are conserved in the pathogen that can have the potential to be recognised in a plant system by pattern recognition receptors (PRRs), triggering a broad but weak host resistance. The second layer of immunity is ETI that induced by recognition, direct or indirect, of pathogen avirulence effectors by host disease-resistance (R) proteins produced by R genes (Wang *et al.,* 2014b; Franco-Orozco *et al.,* 2017).

Abiotic Stresses in Barley

Abiotic stresses are the environmental stresses not caused by an external organism but by physical or chemical environmental pressures. Physical elements include light, water, and temperature. Chemical elements include phytotoxic compounds, and nutrients such as sodium in the form of salinity (Shinozaki *et al.,* 2015). These stresses can cause detrimental changes in the metabolism, growth, and development of plants reducing the usable crop yield and, in extreme cases, causing plant death (Sha Valli Khan *et al.,* 2014). Thus, understanding the method of stress sensing and response is critical in protecting plants against these conditions (Cramer *et al.,* 2011; Zhu*,* 2016). Mechanisms of adaptation to stress are usually subdivided into two general categories: avoidance mechanisms like long roots to avoid drought, and tolerance mechanisms such as increased ion transporters in rice to sequester excess sodium into the vacuole (Gao *et al.,* 2007; Shinozaki *et al.,* 2015).

Many abiotic stresses are found in conjunction with other abiotic and biotic stresses. An example of this is the combination of heat and drought stress that has been shown to have specific plant responses separate from the responses to the individual stresses (Mittler*,* 2006). The combination of abiotic stress can also be antagonistic in terms of tolerance mechanisms. An example of this is that of heavy metal toxicity and drought stress, where the mechanism for tolerance to one exacerbates the effect of the other (Barceló and Poschenrieder*,* 1990). As these mechanisms of tolerance to abiotic stresses require increased resources, such as key micronutrients for defence enzymes, nutrient deficiency is an additional concern when using and breeding for abiotic tolerant plants (Mittler*,* 2006). Biological interactions also play a key role in abiotic stress, such as the biotic-abiotic stress interactions detailed in the next sections, as well as interactions with the rhizosphere microbiome such as the role of plant-growth-promoting rhizobacteria in prompting an ‘induced systemic tolerance’ to salt and drought (Yang *et al.,* 2009), and the suggested role of mycorrhizal in drought stress tolerance (Grover *et al.,* 2011).

Climate change holds a particular challenge regarding abiotic stress as changing and increasingly variable environments will cause new abiotic stresses to develop in different regions. These include

fluctuations in temperature, light, water, carbon dioxide and nutrient availability (Sinclair, 1992; Koski, 1996; Newton *et al.,* 2011b; Hatfield *et al.,* 2014). This is compounded by the increased crop demand to feed the increasing population, and thus the estimated average reduction in crop productivity of 65-87% caused by abiotic and biotic stress needs to be addressed (Shinozaki *et al.,* 2015). Varieties that are able to overcome these challenges need to be developed (Wheeler and von Braun, 2013), and genetic mapping poses to be a great tool in identifying stress tolerant loci that can be used in this development (Pereira, 2016).

Nutrient Stress

Nutrient stress in plants can be a result of either a deficiency or excess of micro- or macro-nutrients. Limitation of nutrients in a plant system can often cause permanent damage causing the plant to use other resources less efficiently, resulting in loss of yield (van Maarschalkerweerd and Husted, 2015; Schmidt *et al.,* 2016a). The eight essential micronutrients needed for plant growth are chlorine (Cl), boron (B), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni) and molybdenum (Mo), along with six essential macronutrients nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) (White and Brown, 2010). Toxicity occurs when the concentration of one or more nutrients is too high, so that the subcellular compartmentalization and chelation of these minerals is ineffective, allowing the nutrients to disturb vital systems. Common nutrient toxicities include sodium (Na) and heavy metal toxicity. Common heavy metals that are toxic at high concentrations to plants include cadmium (Cd), mercury (Hg), chromium (Cr), cobalt (Co), lead (Pb), Zn, Fe, Cu, Al, Ni, and Mn, many of these are mainly found in toxic concentrations due to anthropogenic activity (Foy *et al.,* 1978; Maathuis and Amtmann, 1999; Fageria, 2001; Yadav, 2010; Kronzucker *et al.,* 2013).

Interactions with other nutrients may enhance or decrease the level of sensitivity to toxic or deficient nutrient levels. The reason for this positive or negative interaction is dependent on the nutrients involved and can be direct or indirect. An example of an indirect negative interaction is that of N and micronutrients, which is due to increased growth stimulated by N availability causing higher demand on limited micronutrients (Fageria, 2001). An example of a direct effect is the antagonistic effect found

between Mn and Al, with the effect of the toxic concentrations of both together being less than that of the same level individually (Muhammad *et al.,* 2016).

Environmental conditions also play a crucial role in the deficient and toxic effect on nutrients, notably due to differing water levels and pH values in the soils. Waterlogging stress has been shown to cause a deficiency in nutrients such as N, P, K, Mn, Cu, and Zn due to limited oxygen causing inhibition of ATP (Steffens *et al.,* 2005), it has also been shown to cause toxicity to Mn and Fe due to a change in available ion concentration (Mengel and Kirkby*,* 2001). Ion solubility is also affected by the presence or absence of H^+ ions. Increased levels of H^+ ions, low pH, causes an increase in the solubility of Al, Mn, and Fe resulting in toxicity. Corresponding deficiencies occur at high pH. H^+ ions can also outcompete Ca^{2+} ions at concentrated levels, causing a deficiency in Ca (Alam *et al.,* 1999; Horneck *et al.,* 2011).

Barley Breeding

Barley is one of the most heavily produced crops in the world, with current global production at over 140 M tonnes as of 2016. Global average barley yield has more than doubled in the last 50 years, 1,328 to 3,011 kg/Ha between 1961 and 2016, but the total production has remained similar due to a decline in land area used for barley. This yield increase is also seen in the UK where the harvest area reduced by over 50% since the high in 1966, but with less than a 25% drop in production (FAOSTAT).

This average yield increase is partially due to classical breeding techniques of the 20th century. During this time commercial plant breeding of crops was established and used targeted selective breeding coupled with techniques such as new statistical methods, cytoplasmic male sterility, embryo rescue, mutagenesis (chemical, radiation and transposons), and backcrossing – creating new high-yielding varieties (HYVs) (Sneep *et al.,* 1979; Borém and Milach*,* 1998; van de Wiel *et al.,* 2010; Shu *et al.,* 2012). The HYVs of maize, wheat and rice from the International Maize and Wheat Improvement Centre (CIMMYT) in Mexico and the International Rice Research Institute (IRRI) in the Philippines, developed during the period in and around world wars, resulted in large yield increases that then spread around the world in the 1960s, along with other innovative farming techniques, creating the

Green Revolution (Davies, 2003; Evenson and Gollin, 2003). However, HYVs of barley were not commercially bred until the late 1970s, as barley was often grown under harsh, low rainfall environments by poorer farmers (Aw-hassan *et al.,* 2003).

Current breeding targets to increase barley yield encompasses a wide range of traits, directly and indirectly related to grain production (Friedt *et al.,* 2010). In the UK, lines are assessed by the Agriculture and Horticulture Development Board (AHDB) on up to 13 different characteristics for spring barley, in addition to the assessment of yield – which itself is subdivided by the growth region (AHDB, 2018). These characteristics become breeding targets for companies in a drive to achieve varieties that make it onto the AHDB recommended list, thus focusing the breeding for growing on favourable lands and possibly away from the development of robust crops for less favourable lands and conditions, both of which are likely to increase with climate change. Due to the varying environments around the world, different countries breed for different traits. Abiotic stress is a big concern, thus the Grains Research and Development Corporation in Australia have outlined tolerance to drought, salinity, manganese deficiency, and aluminium toxicity as targets (Friedt *et al.,* 2010).

Agricultural practices rely on healthy and vigorous crops to provide economic productivity. Ongoing global climate change is causing changes in the local environments that threaten the optimum health and vigour of many crops, and thus the food security of nations (Nelson *et al.,* 2010). Robust crops that can adapt to the changing environments need to be developed to be able to cope with fluctuations in temperature, light, water, carbon dioxide and nutrient availability, along with the fluctuation in associated pests and diseases (Sinclair, 1992; Koski, 1996; Newton *et al.,* 2011b; Hatfield *et al.,* 2014). The need to breed pre-emptively for these situations is being highlighted, with traits and lines of interest being identified (Newton *et al.,* 2011b; Ingvordsen *et al.,* 2015; Atlin *et al.,* 2017).

Modern plant breeding, to breed for the future varieties, encompasses a wide range of molecular biological and genomic techniques including; reverse breeding using double haploidy, genetic modification, and marker assisted selection (Barabaschi *et al.,* 2016; Glenn *et al.,* 2017).

Marker-assisted Breeding

Classical breeding works by breeding individuals selected based on the phenotypic data, this was aided with the use of phenotypic and protein markers in the beginning of the 20th century (Lammerts van Bueren *et al.,* 2010). These phenotypes may be based on genetic variations. Regions in the genome that correspond with these phenotypes are called quantitative trait loci (QTLs) and may be associated with genes, or clusters of genes, which are responsible for this change in phenotype. In the 1980's DNA markers were developed, which are regions of genetic difference between individuals being tested and can be selected based on the similarity e.g. between species, cultivars, or individuals (Collard *et al.,* 2005).

The use of multiple markers allows a linkage map to be developed to help identify chromosomal regions containing QTLs controlling variation in the trait of interest. The use of linkage maps aids the identification of QTLs associated with Mendelian traits but is less effective for traits with multiple genes and polyploid crops. Fine mapping addresses some of these problems and is commonly undertaken using a genome-wide association study (GWAS). Typically, the markers used in this are single nucleotide polymorphisms (SNPs), using a diverse population which will have a large number of historical recombination events. Such analyses uses the principle of linkage disequilibrium (LD), meaning unmapped causative SNP and indels (insertions or deletions of base pairs) will be closely associated with the SNP markers nearby compared to those further away (Visscher *et al.,* 2012; Xiao *et al.,* 2017). Recent developments in high-throughput sequencing and genotyping has allowed for the identification of a vast number of SNPs in the elite cereal crops such as maize (Huang and Han, 2014), wheat (Rimbert *et al.,* 2018), and barley (Bayer *et al.,* 2017), this allows a greater detail in identifying the position of the associated loci. Markers that are highly associated with a certain trait can then be used in MAS as mentioned above. The technique MAS uses markers associated with certain traits to select the progeny that are most likely to contain suitable alleles from a segregating breeding population, allowing the removal of unfavourable lines without the wasted resources of growing them until a visible phenotype is seen (Nadeem *et al.,* 2018).

Project Focus

The soil conditions in the regions that Bere barley grows varies widely, with many areas supporting crops on highly alkaline soils (Martin *et al.,* 2008b) with associated manganese deficiency. Other regions such as North-east Caithness and the Northern Isles of Scotland, where Bere lines grow, can experience salt-laden winds year-round causing salt stress (Dry and Robertson*,* 1982). Material collected from these areas, by the University of the Highlands and Islands and kept in the James Hutton Institute Spring Barley Landrace Collection (JHI-SBLC), thus offers a promising genetic potential in the breeding resistance/tolerance to biotic and abiotic stress in barley plants for commercial growth. The main aim of this investigation is to identify novel stress resistance/tolerance in Bere lines, other landraces and old cultivars (Table 2), for both abiotic and biotic stresses, and to identify the regions of the genome contributing to the control of this. This will be done by the adaption and implementation of methods to screen individual lines within the landrace collection, isolating genotypes that could provide breeding material for developing elite cultivars that have increased resistance to biotic and abiotic stresses.

The two abiotic stresses that will be tested are Manganese (Mn) and salt. The tests of Mn stress will be looking for lines that have high Mn use-efficiency in a hydroponic system. Alongside this the effect of environmental Mn on the concentration of Mn in the shoot tissue for different lines/cultivars will be analysed. The tests of salt stress will be looking for lines that have a high tolerance to elevated salt levels, specifically sodium, assessing the immediate affects via the rate and early growth during the germination phase, and the longer-term effects based on the growth and development of more mature plants. The biotic stress that will be tested is the diseased caused by the infection with *Rhynchosporium commune*. Assessing the symptoms via the spread of the lesion, alongside the further assessment of rhynchosporium in field trials. This aim will be aided by using genotypic data to perform GWAS' that can be used to isolate molecular markers associated and identify candidate genes of interest. The secondary aim is to identify how these stresses interact and how these interactions differ between the lines/cultivars, this will be undertaken using the selected lines from the previous aim.

Table 2) Lines/cultivars from the JHI-SBLC that were grown up to collect seed from. Bere lines are highlighted in bold, old cultivars are highlighted in italics and underlined, as identified in the JIC-GRU SeedStor (Horler *et al.,* 2017).

Afghan 1169	*Binder-M08*	Hen Gymro-163	Padstow-189
Aramir-M08	*Bonus-127*	Hen Gymro-164	Pembroke-190
Archer-M08	Bowman	Hen Gymro-165	*Plumage Archer Selection-M08*
Aurore-107	Burtons Malting-128	Hen Gymro-166	*Plumage-192*
Balder-108	*BW 902*	Hen Gymro-167	*Plumage-193*
Bavaria	*Camton-129*	Hen Haidd Enlli	*Plumage-M08*
Beavans 35/51-110	*Carslberg-M08*	Hen Haidd Eulii-168	Prior-195
Beavans 35-109	Chevalier D10-130	Hen Hardd Eulii 78 A	*Prize Prolific-196*
Bere (Mr SO)-121	Chevalier-M08	Hen Hardd Eulii 78 B	*Proctor*
Bere (Scots)-122	*China Huang Yen*	Hen Hardd Eulii 78 C	*Proctor-M08*
Bere 23 A	Common-132	*Hindukusch*	*Rene Guillemart-197*
Bere 23 B	Common-218	*HSX07-15*	Rigel-198
Bere 23 C	Cornish-133	*HSX07-20*	Rigel-199
Bere 24268 A 71	*Craigs Triumph (SSRPB)-135*	*HSX07-26*	**SASA 27 A Bere North Uist**
Bere 25 A	*Craigs Triumph B8(8)-136*	*Irish Archer-169*	Scotch Annat 4812
Bere 2962 (AB)	*Craigs Triumph-134*	*Irish Goldthorpe-170*	Scotch Annat-200
Bere 37 A 14	*D.K.S. Binder-137*	*Irish Goldthorpe-171*	Scotch Common-M08
Bere 39 A 16 Berneray	*Danubia*	*Irish Goldthorpe-172*	Scottish Annat 8585
Bere 43 A 21	Donegal landrace-138	*Irish Goldthorpe-222*	Scottish Annat-202
Bere 44 A 22	*Earl-139*	*Isaria*	Scottish Common 28303
Bere 45 A 23	*Earl-140*	*Japan Kitagawa Chobo*	Scottish Common 3584
Bere 47 A 25	Early Welsh-141	*Kenia-M08*	Scottish Common 7083
Bere 4828 A 63	Early Welsh-142	*L92-174*	Scottish Common 7683
Bere 49 A 27 Shetland	Eire Six Row-143	Laevigatum-175	Skadu Local "Oldings"
Bere 52 A 30	Eire Six Row-220	*Lawina*	*Spratt Archer 37/6/3-205*
Bere 53 A 31	Floye	*Lenta-176*	*Spratt Archer-M08*
Bere 55 A 33	*Gartons Archer-144*	Long Eared Nottingham-177	*Spratt-M08*
Bere 55C 33	Glasnevin 1-145	Long John Grant-178	St Davids-206
Bere 58 A 36 Eday	*Gold-146*	*Maja-179*	*Standwell-207*
Bere 59 A 37 Uist	*Golden Archer-147*	*Millenium-219*	*Standwell-208*
Bere 60 A	Golden Drop-148	Morayshire Gold 7009	*Stat -Old 14*
Bere 7045 (AB)	*Golden Melon-149*	Morayshire Gold-180	Streatly-209
Bere 8-125	*Golden Pheasant-150*	*Morex*	Swanneck-210
Bere A 3962 62	*Golden Promise-M08*	*Nepal 92 BN-1*	Swanneck-211
Bere-112	*Goldfield-151*	*New Cross-181*	Swannek-212
Bere-113	*Goldfield-152*	*NFC Tipple*	Swannek-213
Bere-114	Goldthorpe-153	Northumberland Rogue-182	*Tibet37*
Bere-115	Goldthorpe-154	Old Cromarty-183	Tiree six row 12 (AB)
Bere-116	Gotlands-156	Old Irish-184	Tiree six row 12 A
Bere-118	*Gull-158*	Old Irish-221	Vollkorngerste-214
Bere-119	Haidd Garw-159	Old Wilts Archer-185	*Webbs Binder-215*
Bere-120	*Hanna-M08*	Old Wiltshire Archer-187	*Webbs Burton Malting-216*
Bere-155	*Heines Hanna*	Old Wiltshire-186	*Webbs Naked 2-Row-217*
Bere-223	Hen Gymro-161	*Opal-188*	*Westminster*
Bere-M08	Hen Gymro-162	*Optic*	*Zephyr-M08*

Assessing the variation in manganese use efficiency traits in Scottish barley landrace Bere (*Hordeum vulgare* L.)

Introduction

Whilst barley has displayed a greater Mn use efficiency than other temperate cereal crops (Marcar and Graham, 1987) a lack of available Mn still causes major problems in barley agronomy worldwide including areas with: a) high organic matter such as peaty soils in the UK (Jiang and Ireland, 2005) and the Great lakes area of the USA (Adriano, 2001), or loam soils in Alberta and Ontario in Canada (Reid and Webster, 1969); b) poorly draining and coarse textured soils in Sweden, the Atlantic Coastal Plains of the USA, Scotland in the UK (Goldberg *et al.,* 1983), and the Netherlands, the latter also containing poorly draining clay soils (Henkens, 1958); and c) calcareous soils of Northern China and Australia, causing up to 75% yield reduction in Southern Australia (Graham *et al.,* 1982). These conditions are found in combination in Danish soils in Northern and Western Jutland, further affected by over-liming (Steenbjerg, 1935; Reuter *et al.,* 1988). Alkaline and calcareous soils can have a reduced Mn availability as the nutrient is in the Mn(III) and Mn(IV) forms, bound and precipitated as oxides and dioxides (Tisdale and Nelson, 1956; White and Greenwood, 2013). This conversion takes place because Mn availability is controlled by H^+ concentrations (pH) and redox potential. The high pH of the alkaline soils (low H^+) drives the reverse reaction of $MnO_2 + 4H^+ + 2e^- \rightleftarrows Mn^{2+} + 2H_2O$, limiting the amount of available Mn (Blake *et al.,* 1999; Porter *et al.,* 2004; Aciego Pietri and Brookes, 2008).

Manganese is found in multiple forms based on its oxidation state, with three main states being associated with biological systems. The most stable state, Mn(II), is the most soluble in soil and exists as Mn^{2+}. Manganese (III) and Mn (IV) are insoluble species often present bound in the forms of Mn(III) oxide (Mn_2O_3) and Mn dioxide (MnO_2), respectively (Millaleo *et al.,* 2010). In plant systems Mn plays an important role in the function of multiple enzymes and other proteins. Manganese has a key and crucial role as a catalytically active metal in the photosystem II (PSII) oxygen evolving complex (OEC) within chlorophyll, where it catalyses the water-splitting reaction (Schmidt *et al.,* 2015). Other

important biological uses for Mn include its role in NAD-malic enzymes, oxalate oxidase enzymes, glycosyl transferase, and proteins involved in the shikimic acid pathway, occurring in approximately 35 enzymes in total (Hänsch and Mendel*,* 2009; Williams and Pittman*,* 2010). Of these enzymes, three cannot replace the Mn component (Burnell*,* 1988), including: Mn superoxide dismutase (Bowler *et al.,* 1991; Poage *et al.,* 2011), oxalate oxidase (Requena and Bornemann*,* 1999), and as a catalytical Mn cluster (Schmidt *et al.,* 2016b) in the OEC of PSII mentioned above (Ono *et al.,* 1992; Barber*,* 2004). Manganese deficiency has been shown to cause a considerable reduction in PSII supercomplex quantity (Schmidt *et al.,* 2015), whilst retaining OEC protein sub-units such as PsbP and PsbQ (Schmidt *et al.,* 2016b).

Manganese-deficiency is a large problem to global crop production, as is Mn-toxicity (White and Greenwood*,* 2013). When the soil is acidic the forward reaction of $MnO_2 + 4H^+ + 2e^- \rightleftarrows Mn^{2+} + 2H_2O$ is driven and the Mn in the soil becomes available (Porter *et al.,* 2004). In soils rich in organic matter, coupled with a low pH, the availability of Mn^{2+} is in excess and can cause toxicity in the plant. This excess causes an inhibition of respiration & photosynthetic functions, as well as a reduction in chlorophyll content and synthesis of nitrogen and protein (Demirevska-Kepova *et al.,* 2004). The problem of Mn-toxicity in acidic soils can be corrected by increasing the soil pH via the application of Ca and Mg rich materials, such as chalk or limestone (White and Greenwood*,* 2013). As stated previously, the redox potential also affects the availability of Mn in the soil. When the reducing processes dominate during anaerobic conditions, such as waterlogged or flooded soils, the reaction is driven as it is in acidic environments, causing large levels of available Mn. This large concentration of Mn^{2+} has the same effect as in acidic soils, thus waterlogged plants often exhibit Mn-toxicity. This available Mn often rapidly becomes unavailable once the soil is aerated upon draining (Mengel and Kirkby*,* 2001; Huang *et al.,* 2015).

The symptoms of this deficiency are indicated first by inter-veinal bleaching of the middle leaves, followed by a spread of this bleaching and development of brown rimmed blotches (Figure 2). The leaves eventually die off, with substantial tiller death. Affected plants will produce fewer and smaller heads, thus reducing yield; long periods of deficiency can cause plant death (Department of

Agriculture and Food Western Australia, 2015). The visual symptoms of this deficiency are usually quite delayed and thus a timely rectification cannot always be achieved. One method of early detection by analysing the Chlorophyll a fluorescence induction kinetics has been discovered which will aid in the treatment of this deficiency (Schmidt *et al.,* 2013). The deficiency of Mn in a plant subsequently affects pathogens, often increasing susceptibility. Barley grown with adequate Mn, compared to those grown under Mn-deficiency, have been shown to have an increased resistance to a range of pathogens including *B. sorokiniana* – leaf spot, *Fusarium spp*., *Pyrenophora gramineacan* – barley stripe (Gleń *et al.,* 2013), *Blumeria graminis* – mildew; with further diseases found in other cereals (Huber and Wilhelm, 1988) such as *Gaeumannomyces graminis* in wheat – Take-all (Pallotta *et al.,* 2000).

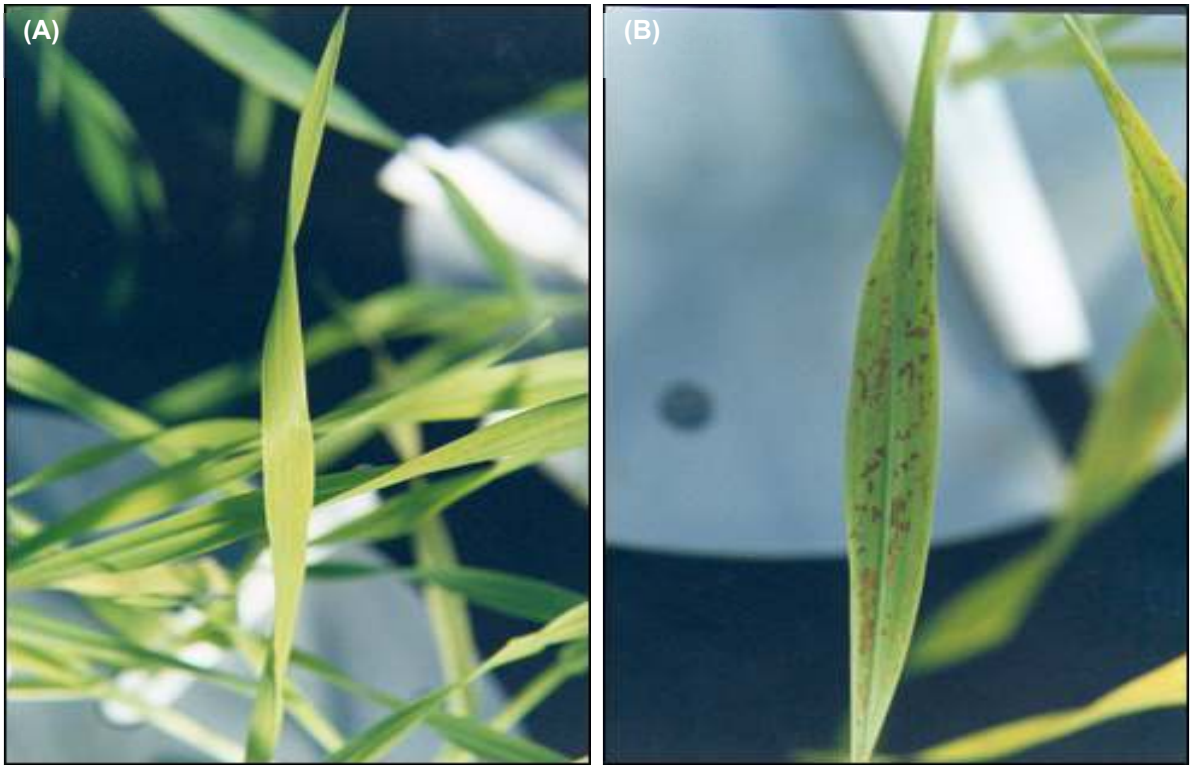


Figure 2) Symptoms of Mn deficiency in barley showing; **a**) early symptoms of interveinal chlorosis, and **b**) later symptoms of necrotic brown spots. Sourced from Schmidt *et al.* (2016a).

Chemical correction of Mn deficiency is limited as Mn supplemented fertiliser is inefficient due to the conversion of the applied Mn in soil into Mn oxides. Foliar application has been shown to be more effective, but has a significant financial cost that makes it expensive to many farmers growing on deficient soils (Schmidt *et al.,* 2013). The best results of fertilisation are seen when both soil and foliar fertilisers are used in combination (Reuter *et al.,* 1973; Pallotta *et al.,* 2000). Manganese-

supplemented soil fertiliser can have increased efficiency in calcareous soils when combined with soil acidification, using sulphur fertiliser or urea (Shuman, 1998; Fageria, 2008; White and Greenwood, 2013).

Pedas *et al.* (2005) observed considerable variability in high-affinity Mn influx kinetics between barley genotypes resulting in differing Mn efficiencies. No differences were observed in the low-affinity Mn influx kinetics in the same study. To date only one plasma membrane-localised Mn^{2+} transporter protein encoding gene has been identified in barley, Iron Regulated Transporter 1 (*Hv*IRT1). Pedas *et al.* (2008) demonstrated that the HvIRT1 gene was up-regulated in Mn deficient soils, with up to 40% greater expression than in Fe-deficient soils, thus it could be an important factor in breeding for Mn efficient barley. Differences in IRT have been shown between species highlighting regions of little to no conservation, these differences could explain the dissimilarities in uptake efficiency such as the differences in Zn uptake between the Arabidopsis and barley IRT1 (Pedas *et al.,* 2008). Whilst the differences in the high-affinity Mn influx kinetics observed by Pedas *et al.* (2005) have been shown not to be due to genetic differences in the IRT1, it has been suggested that they could still be due to different isoforms of the Mn transporters rather than the level of expression (Pedas *et al.,* 2008). Schmidt *et al.* (2016a) also suggested that plants do not rely on a single mechanism of Mn transport for uptake. Additionally, an early maturing Japanese cultivar Amagi Nijo (Tsuda *et al.,* 1979), has two further loci identified that are associated with an increase Mn use efficiency; Mn Efficiency Locus 1 (*Mel1)* identified by Pallotta *et al.* (2000) and a putative second locus around the RFLP marker *Xwg645* (Lloyd, 2000; McDonald *et al.,* 2001). Physiological difference may also help account for increased Mn use efficiency as it is possible that root length and architecture, together with the rhizosphere, effect Mn accumulation due to an increase in fine root hairs triggered by exudate release as seen in Alfalfa (Gherardi and Rengel, 2004) and suggested in barley (George *et al.,* 2014).

The aims of this study were to confirm if there is an inherent Mn use efficiency in the Bere lines, as well as identifying individual lines that have high use efficiency. This data was used to identify differences in Mn accumulation in the leaf tissue and identify genomic regions associated with this trait and speculate on any candidate genes in these regions.

Results

Landrace Screen

1.1.1.3. Sub-category Score Analysis

There were significant differences in chlorophyll fluorescence between the Mn concentrations, lines/cultivars, and interaction of these variates (all $p<0.001$) when lines were grouped by subcategory. The Bere lines had a greater chlorophyll fluorescence in low Mn concentrations compared to the other landraces & elites (Figure 3).

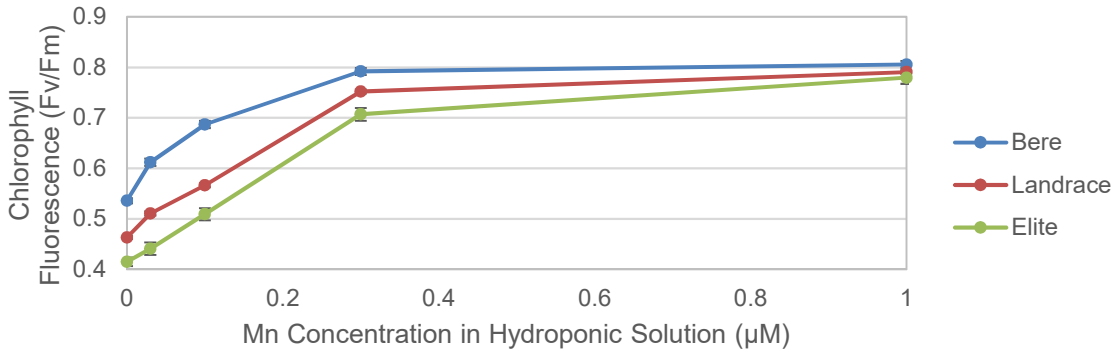


Figure 3) Chlorophyll fluorescence for 140 lines/cultivars of barley divided into three subcategories Bere, other landraces and elites (n=36, n=94, and n=10, respectively) over five differing Mn concentrations. Error bars represent the standard errors in positive and negative directions.

The FR showed the extent to which the three sub-categories were affected over these Mn concentrations. The Beres showed the smallest FR, the other landraces had approximately 50% greater reduction and the elites the largest reduction of over 80%, compared with the Bere lines (Figure 4).

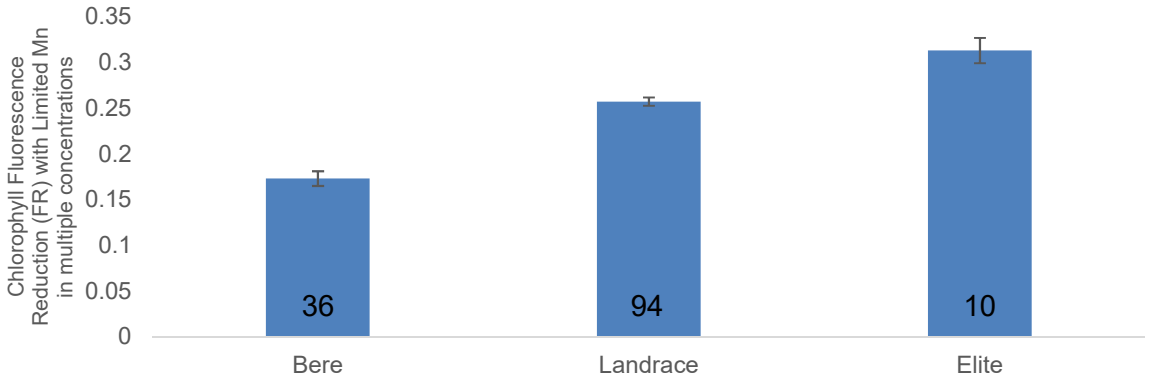


Figure 4) Chlorophyll Fluorescence Reduction for 140 lines/cultivars of barley divided into three, unequal, sub-divisions to compare the relative Mn deficiency in each; with a low FR indicating less change of Chlorophyll Fluorescence from the optimum. Error bars represent the standard errors in positive and negative directions. The number of lines/cultivars collated is noted at the base of each bar.

1.1.1.4. Individual Fluorescence Reduction Analysis

Separating the groups into their individual lines, there were significant differences between the Mn concentrations, lines/cultivars and interaction of these treatments ($p<0.001$). The FR shows the extent to which the 140 lines/cultivars were affected over the range of Mn concentrations (Figure 5). The greatest FR, or the lowest Mn use efficiency, was for the elite cultivar Scholar with a FR of 0.35. As noted above, the elite cultivars had amongst the greatest FR within the population tested, the smallest FR in an elite cultivar was Westminster at 0.27, still in the upper third of the population in terms of FR. The smallest FR overall was Bere 24268 A 71 at 0.07, 80% less than Scholar. The 19 smallest FRs were all Bere lines with FRs less than 0.17. The Bere lines with the greatest FR were Bere 8-125 and Bere 2962 (AB), each having a FR of 0.31 making them comparable to some of the elite cultivars. The lines in the landrace sub-category had the greatest range of FR. The line Stat -Old 14 had the second greatest FR at 0.34, comparable to the elite cultivar Scholar. The line with the smallest FR was Webbs Burton Malting-216, 20th lowest scoring line, with a FR of 0.17, half that of Scholar, and the only non-Bere line in the top 20 lines with the smallest FR.

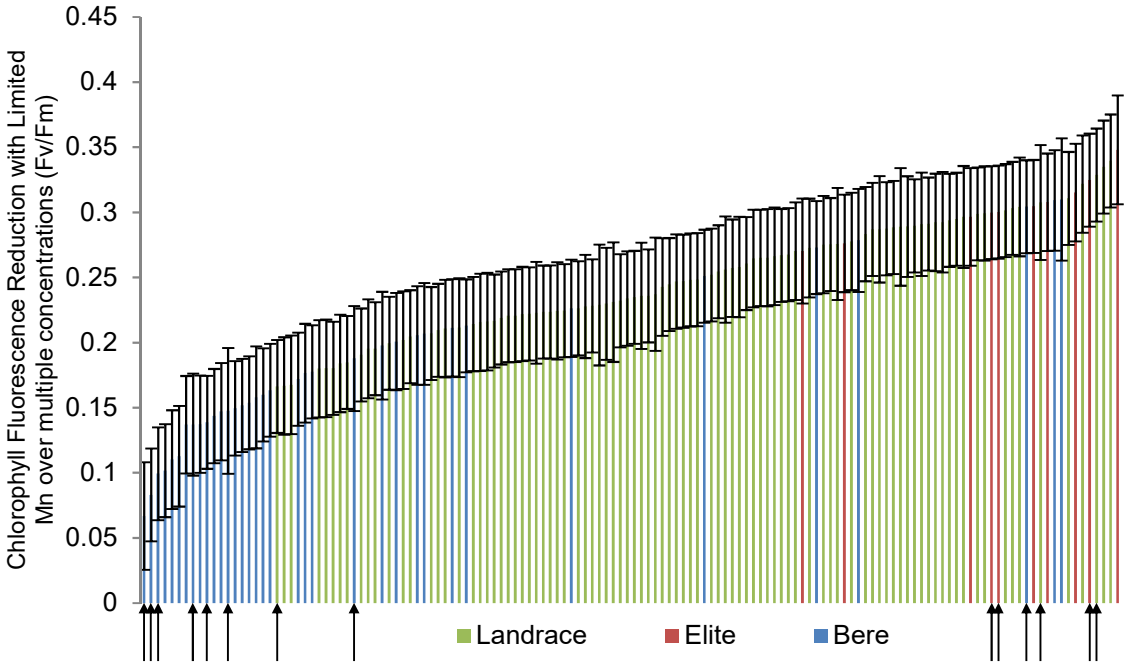


Figure 5) Chlorophyll Fluorescence Reduction 140 lines/cultivars of barley, colour coded for the sub-divisions, comparing Mn deficiency in each the– with a low FR indicating less change of Chlorophyll Fluorescence from the optimum. The arrows indicate the lines selected to be used to measure the Mn concentration in the leaf tissue below. Error bars represent the standard errors in positive and negative directions.

1.1.1.5. Genotype Individual Mean Analysis

Analysis of the mean fluorescence data (not shown) showed a trend of increasing fluorescence with increasing Mn concentration in the hydroponic solution for each line/cultivar as expected from the FR results, but showed some divergence. Bere 47 A 25 and Bere 58 A 36 Eday show large increases in chlorophyll fluorescence but had a smaller fluorescence at 0 µM Mn, with fluorescence measurements becoming comparable to the smallest FR Bere lines at an Mn concentration of 0.03 µM. The worst performing Bere lines at 0 µM – Bere-118, Bere A 3962 62, and Bere 58 A 36 Eday – had smaller chlorophyll fluorescence measurements, but greater increases in chlorophyll fluorescence with increases in Mn concentration. Similar differences in chlorophyll fluorescence with changing Mn can be seen between elite cultivars. Optic had the smallest differences in chlorophyll fluorescence with increased Mn of the elites, but Waggon never gets to optimal chlorophyll fluorescence within the 0-1 µM range tested, not passing 0.72 at 0.3 µM, despite having the greatest chlorophyll fluorescence for an elite cultivar at 0 µM.

1.1.1.6. Genome-Wide Association Study (GWAS) Analysis

From the 37242 markers used, 10725 were removed as having low minor allele frequency and a further 32 because of a low call rate. Of the 142 lines used 13 were excluded due to high heterozygosity, and a further 10 due to being identical by state.

The QQ plots showed that the MLM model for the 0 µM mean had the smallest deviation from the expected null distribution. The Manhattan MLM plot for the 0 µM mean (Figure 6a) displayed multiple loci of interest, the most statistically significant association was on the distal end of chromosome 2HL along with other associations at 5HL. Two additional associations at the distal end of 5HS and the proximal end of 6HL were identified in the Manhattan MLM plot for FR and area under the curve (AUC) (Figure 6b & c).

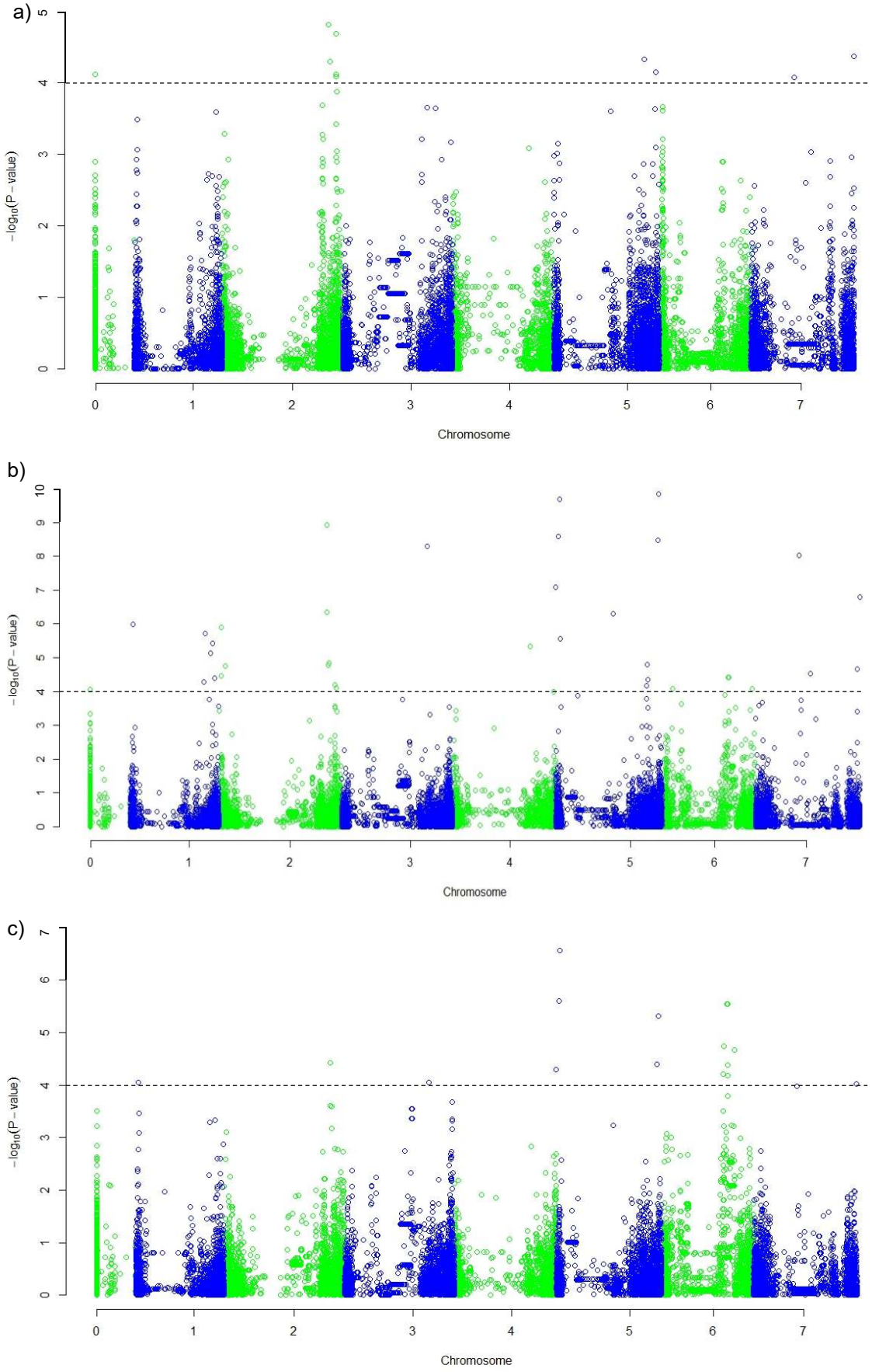


Figure 6) Manhattan plots of a Genome-Wide Association Study undertaken using a Mixed Linear Model on the **a)** 0µM Mn average, **b)** FR and **c)** AUC data generated using an ANOVA. Depressions in marker significance observed in the centre of each chromosome are due to reduced marker density around the centromere of the physical map.

In the 0 μM mean data 14 significant markers were identified ($p<0.0001$), all with an effect of 0.025-0.032 Fv/Fm (Table 3). Of these 9 were on the locus located distally on 2HL between 687.83-725.12 Mb. Within this there were three localised regions: 687.83 Mb and 724.94-725.12 Mb each with four significant markers along with one marker at 677.31 Mb. The other markers were in separate locations including the distal end of 7HL, and the centre of 5HL. In the region identified on chromosome 2HL (687.83-725.12 Mb) there are many associated genes, with 15 genes identified as potential candidates (Table 4). The 4 sequential markers were found to all be located within a gene encoding for a KS protein with metal-binding Terpene synthase domain (HORVU2Hr1G099480). Other candidate genes in this area encode for: 1) a 3-phosphoglycerate dehydrogenase, 2) a Serine/threonine-protein, 3) a MATE efflux family protein, 4) a yellow stripe-like protein, 5) two heavy metal ATPase', 6) five transporter proteins, for K, Zn, Sulphate, or amino acids, and 7) three serial Photosystem I P700 chlorophyll a apoprotein. Lone markers positioned on 5HL and 7HL were contained within/next to a Photosystem II protein and a Serine/threonine-protein kinase, respectively.

Table 3) The statistically significant markers found in the GWAS of the 0 μM Mn data, with the chromosome number, position on the physical map, statistical significance, and the effect of the marker (increase in the Fv/Fm ratio) listed.

Marker Name	Chromosome	Position (Mb)	P-value	Effect
JHI_Hv50k_2016_110885	2H	677.31	1.55E-05	0.0296
JHI_Hv50k_2016_113750	2H	687.83	5.07E-05	0.0274
JHI_Hv50k_2016_113753	2H	687.83	5.07E-05	0.0274
JHI_Hv50k_2016_113754	2H	687.83	5.07E-05	0.0274
JHI_Hv50k_2016_113755	2H	687.83	5.07E-05	0.0274
JHI_Hv50k_2016_128224	2H	724.95	7.72E-05	0.0281
JHI_Hv50k_2016_128255	2H	724.97	2.07E-05	0.0312
JHI_Hv50k_2016_128280	2H	724.97	7.72E-05	0.0281
JHI_Hv50k_2016_128407	2H	725.12	8.22E-05	0.0267
JHI_Hv50k_2016_323762	5H	573.35	4.77E-05	0.0301
JHI_Hv50k_2016_355863	5H	648.01	7.21E-05	0.0253
SCRI_RS_167383	7H	275.46	8.54E-05	0.0265
JHI_Hv50k_2016_518726	7H	654.39	4.29E-05	0.0263
12_30351	U	-	7.72E-05	0.0281

Looking at the Manhattan MLM plot for the AUC and FR there were also two regions in the centre of 6HL, 391.68-394.17 Mb with five significant markers and 413.26-417.83 Mb with nine. These two QTLs contained three genes of interest, encoding for a yellow stripe-like protein, an Al resistance family protein, and a Nramp1 (HORVU6Hr1G061740.1) (Table 4).

Table 4) A list of the identified genes of interest with regards to manganese use efficiency, along with their position, and the genetic annotation.

Gene Name	Chr	Position (Mb)	Annotation
HORVU2Hr1G096930.1	2HL	677.16	Heavy metal ATPase 5
HORVU2Hr1G097010.8	2HL	677.26	Copper-transporting ATPase 1
HORVU2Hr1G099170.1	2HL	686.91	Photosystem I P700 chlorophyll a apoprotein A1
HORVU2Hr1G099180.1	2HL	686.91	Photosystem I P700 chlorophyll a apoprotein A1
HORVU2Hr1G099190.1	2HL	687.03	Photosystem I P700 chlorophyll a apoprotein A1
HORVU2Hr1G099480.13	2HL	687.83	KS protein with a metal-binding Terpene synthase domain
HORVU2Hr1G099530.1	2HL	687.96	Cationic amino acid transporter 8
HORVU2Hr1G099680.1	2HL	688.06	Amino acid transporter 1
HORVU2Hr1G099810.14	2HL	688.52	Potassium transporter family protein
HORVU2Hr1G099860.1	2HL	688.60	YELLOW STRIPE like 7
HORVU2Hr1G112090.3	2HL	724.96	Serine/threonine-protein kinase
HORVU2Hr1G112150.1	2HL	725.00	MATE efflux family protein
HORVU2Hr1G112230.2	2HL	725.23	Zinc transporter 8
HORVU2Hr1G113050.1	2HL	727.21	Sulphate transporter 91
HORVU2Hr1G113180.3	2HL	727.57	D-3-phosphoglycerate dehydrogenase
HORVU5Hr1G084800.1	5HL	573.35	Photosystem II protein N
HORVU6Hr1G059420.2	6HL	392.46	YELLOW STRIPE like 7
HORVU6Hr1G061740.1	6HL	413.26	Metal transporter Nramp1
HORVU6Hr1G061880.1	6HL	414.17	Aluminium resistance family protein
HORVU7Hr1G121690.1	7HL	654.38	Protein kinase superfamily protein

Of the genes identified the photosystem associated proteins are common, comprising of 0.28% of the 73586 genes listed in BARLEX the barley genome explorer (Colmsee *et al.,* 2015) at 76 and 130 genes genome-wide associated with photosystem I and II, respectively. The protein kinases are the most common, as expected, with genes annotated as Serine/threonine-protein kinases and Protein kinase superfamily proteins representing 0.45 and 0.78% of genes at 328 and 572, respectively. The specific transporters identified here range from MATE efflux family proteins at 0.13% (94 genes) to Nramp proteins at only 0.01% (8 genes): with Amino acid, Potassium, Zinc, and Sulphate transporters in-between with 59, 38, 32, and 22 genes each, respectively. The ATPases identified in this study, Heavy metal and copper-transporting, are less common with only 3 and 14 genes, respectively. The other KS proteins are a very common annotation, but only 35 genes have a Terpene synthase metal-binding domain, representing 0.05% of genes. Yellow Stripe protein encoding genes are also relatively uncommon with only 29 genome wide, or 0.04%. The remaining identified genes – D-3-phosphoglycerate dehydrogenase and Aluminium resistance family protein – are amongst the least common with only 5 and 3 genes each, respectively.

Manganese Quantification in Shoot

The mineral concentration for the 14 lines/cultivars selected from the screen above (identified by arrows in Figure 5) showed significant differences ($p<0.005$) between lines/cultivars for all elements tested, with the exception of nickel. The element ^{55}Mn was the only element that had significant differences between different Mn concentrations ($p=0.003$) the interaction between Mn concentrations and lines/cultivars ($p<0.001$). Two other elements also had interactions of note: ^{139}K and ^{24}Mg ($p=0.037$ and 0.053, respectively).

Based on the differences seen in the Mn concentrations, when grown at 0 and 1 μM $MnCl_2$, and the chlorophyll fluorescence of plants grown in 0 μM $MnCl_2$ (Figure 7), three separate groups can be identified in the subset of lines/cultivars analysed, diverging broadly along the criteria that they were selected on. The group with greatest Mn use efficiency – Bere 24268 A 71, Bere 45 A 23, Bere 47 A 25, and Bere 59 A 37 Uist – showed Mn concentration between 175-270 mg kg^{-1} DW when grown in 1 μM $MnCl_2$ hydroponic solution and 15-22 mg kg^{-1} DW when grown in 0 μM $MnCl_2$ hydroponic solution. They also showed the greatest chlorophyll fluorescence, retaining more than 95% of the maximum quantum yield of photosynthesis of 0.83. The second group had moderate Mn use efficiency and had Mn concentrations between 130-170 and 9-12 mg kg^{-1} DW when grown in 1 and 0 μM $MnCl_2$ hydroponic solutions, respectively, and maintained >75% of the maximum quantum yield of photosynthesis. This group consisted of Bere-155, Bere 25 A, Bere 58 A 36 Eday, and the landrace with the smallest FR, Webbs Burton Malting-216. The last group were Mn inefficient with Mn concentrations between 60-85 and 7-7.5 mg kg^{-1} DW when grown in 1 and 0 μM $MnCl_2$ hydroponic solutions, respectively, and retained less than two-thirds of the maximum quantum yield of photosynthesis. This last group contained the elite cultivars, along with the other landraces and Bere line (Bere A 3962 62) with the greatest FR.

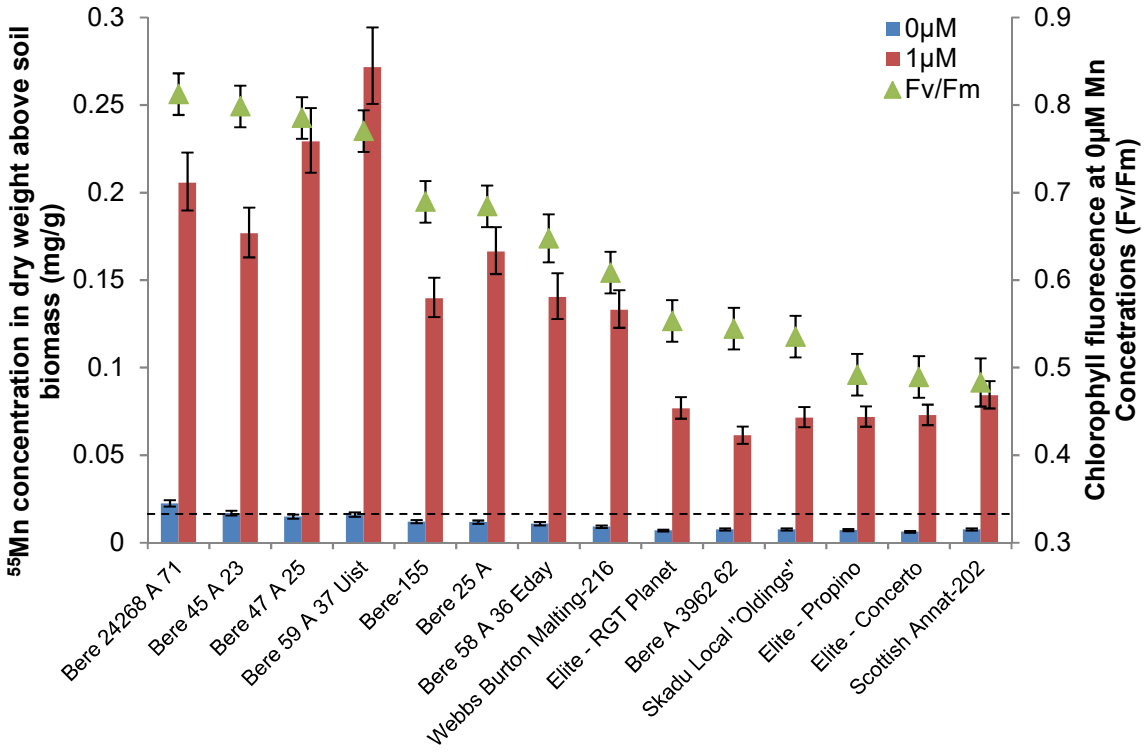


Figure 7) A subset of the population representing Bere, landrace, and elite lines/cultivars over a range in FR. The columns (primary axis) display the mean ^{55}Mn concentrations in the dry weight of the shoot biomass for plants grown in a hydroponic solution of 0 µM $MnCl_2$ (blue) and 1 µM $MnCl_2$ (red); the black dotted line indicate the specified critical deficiency threshold concentration of Mn in leaf tissue of 17 mg kg^{-1} DW as outlined by Reuter *et al.* (1997). The green data points (secondary axis) display the mean chlorophyll fluorescence of the plants grown in a hydroponic solution of 0 µM $MnCl_2$. Error bars represent the standard errors in positive and negative directions.

The 14 selected lines/cultivars grown in a 0 µM $MnCl_2$ concentration had small concentrations of Mn in the biomass, with small but significant differences between the lines/cultivars ($p<0.001$). When the shoot Mn concentration for each individual was compared against the corresponding weight of the shoot biomass it can be seen that there is a weak correlation of decreasing shoot Mn levels with increasing shoot biomass (Figure 8). Statistical analysis of the data with shoot weight as a co-factor shows that this effect does not change the result. The data of the shoot Mn concentrations and the chlorophyll fluorescence of plants grown in 0 µM $MnCl_2$ hydroponic solution were highly correlated, with a significant correlation coefficient of 0.93 (Figure 9a; $p<0.001$). This was greater than the correlation of the shoot Mn concentration of plants grown in 1 µM $MnCl_2$ with the chlorophyll fluorescence of plants grown in 0 µM $MnCl_2$ (with a coefficient of 0.91; Figure 9b), and the correlation of the shoot Mn concentrations of plants grown in the two $MnCl_2$ concentrations (with a coefficient of 0.85; Figure 9c) – both of which were still highly corelated, though the latter was found not to be significant ($p=0.277$).

Lines/cultivars grown in a 1 μM $MnCl_2$ concentration showed large and significant ($p<0.001$) differences in concentrations of Mn in the shoot biomass, between 8-17 times greater than the concentration when grown in the absence of Mn. The four Bere lines that exhibited the greatest Mn use efficiency showed 2.3-3.8 times the concentration of Mn than the elite cultivars, with no sign of Mn toxicity. However, there was no difference between cultivars/lines in the chlorophyll fluorescence of plants grown in 1 μM $MnCl_2$, but there was a negative correlation at this level between shoot Mn concentration and the chlorophyll fluorescence (with a coefficient of -0.81; Figure 9d).

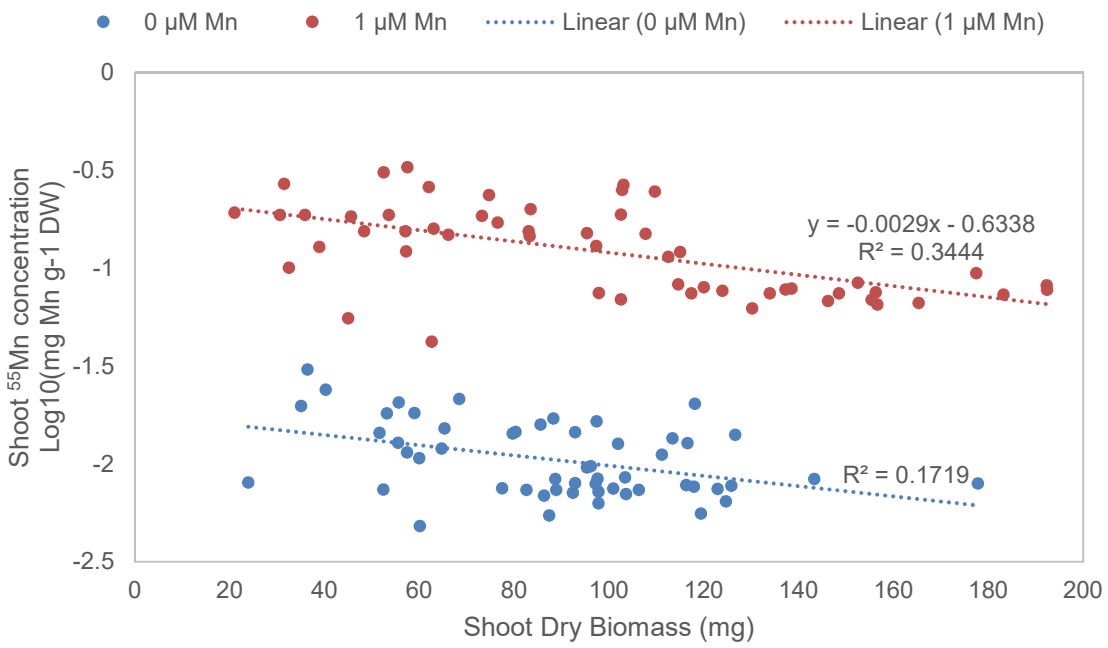


Figure 8) Correlation of Shoot ^{55}Mn concentration and the shoot biomass for each individual; separated into those grown in 0 (**blue**) and 1 (**red**) μM Mn. For each correlation the line of best fit along with the coefficient of determination (R^2) value is given; P-values=0.002 and <0.001, respectively.

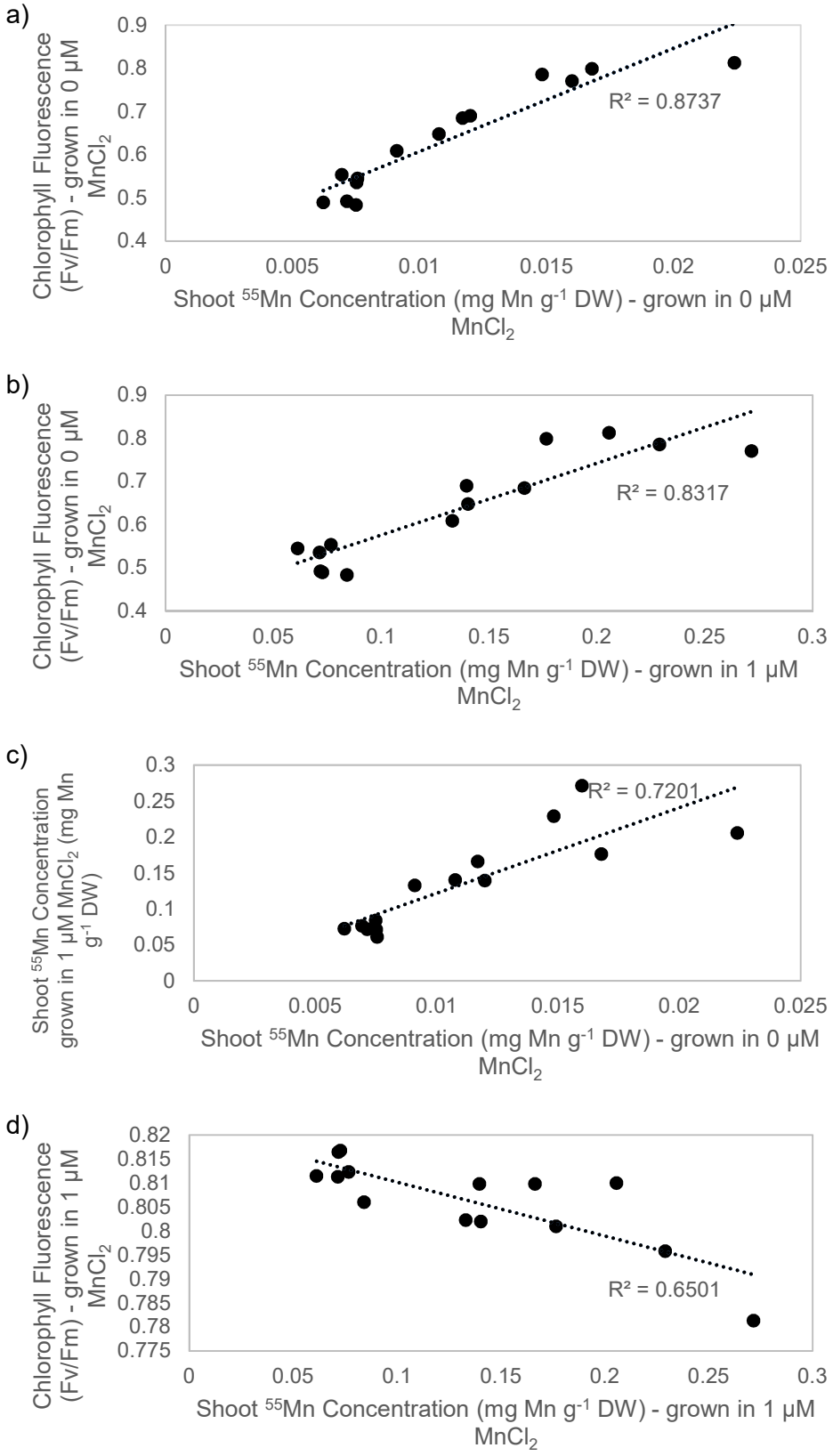


Figure 9) Correlation of averages of the14 lines/cultivars, between the chlorophyll fluorescence when grown in 0 µM $MnCl_2$ and Shoot ^{55}Mn Concentration of plants grown in **a)** 0 µM $MnCl_2$ and **b)** 1 µM $MnCl_2$. As well as the correlation between **c)** the Shoot ^{55}Mn Concentration of plants grown in 0 and 1 µM $MnCl_2$, and **d)** the chlorophyll fluorescence and Shoot ^{55}Mn Concentration of plants grown in 1 µM $MnCl_2$. For each correlation the line of best fit along with the coefficient of determination (R^2) value is given; P-values<0.001 for all bar graph **c** for which =0.277.

Discussion

Manganese deficiency is a problem for marginal lands worldwide, reducing the yield and area of effective crop production (Schmidt *et al.,* 2013). One method of improving the sustainability of plant production, on agricultural soils with limited Mn availability, is by incorporating Mn use efficiency traits into elite crop cultivars. Sources of such Mn-efficiency traits may come from landraces of barley that have developed on marginal soils over many generations (Schmidt *et al.,* 2018). This study validates that Bere lines are a good source of genetic variation in Mn-efficiency and shows that Beres, in general, are superior to other landraces and elite lines in this regard. The study then goes on to identify particular lines and loci associated with this superior Mn-efficiency trait.

Effect on Chlorophyll Fluorescence

The landrace screen provides sufficient evidence to suggest that the majority of the Bere barley lines tested have increased Mn efficiency, compared to the elite cultivars and other landraces tested, in low and no Mn hydroponic systems. This supports and expands on the work undertaken by George *et al.* (2014), Leplat (2015), Brown *et al.* (2017), and Schmidt *et al.* (2018), further identifying Bere lines of interest in regards to Mn use efficiency, including Bere lines that do not show this Mn use efficiency and Bere lines that show Mn use efficiency only at low levels (but not Mn devoid). The investigation also indicates that the elite spring barley cultivars that were included had extreme levels of latent Mn deficiency, thus indicating a need for Mn use efficiency traits within the UK breeding populations. However, other elite cultivars have been shown to have Mn-efficiency such as the Australian *Weeah* barley cultivar (Huang*,* 1996).

Particular lines of interest with regards to Mn use efficiency identified here include Bere 24268 A 71, Bere 45 A 23, and Bere 47 A 25. Lines such as Bere 2962 (AB) and Bere 8-125 are also of interest as they are genetically similar but have a comparably low Mn use efficiency. These two lines have been shown to group away from the main Bere cluster in the genotyping data along with Bere A 3962 62, that has a low chlorophyll fluorescence at 0 µM but average fluorescence with low amounts of

Mn. This suggests that this group may come from a more isolated region of the highlands and islands where Mn deficiency is not a major selective pressure, possibly due to selection on acidic soils.

Effect on Shoot Manganese Concentration

Analysis of the Mn concentration in the shoot biomass showed that an increased level of accumulation of Mn in the biomass corresponded to an increased Mn use efficiency, and that this occurred when there was an adequate supply of Mn in the environment. This accumulation reached a concentration that could be considered above the specified critical toxicity threshold concentration for the highly to moderately Mn efficient lines. The four Bere lines with the greatest Mn use efficiency, along with Bere 25A, rose above the 150 mg kg^{-1} DW critical limit outlined in Reuter *et al.* (1997), and the remaining moderately Mn efficient lines rose above the 120 mg kg^{-1} DW critical limit for Mn toxicity, outlined in MacNicol and Beckett (1985). However, no lines showed toxicity symptoms at the early stages of growth, thus indicating a decreased sensitivity to toxic Mn concentrations in addition to Mn-efficiency under limited Mn conditions.

All lines/cultivars showed large decreases in Mn content when grown in Mn deficient conditions, but Mn efficient Bere lines built up a concentration large enough to avoid the specified critical deficiency threshold concentration of Mn in leaf tissue, which ranges from 11 to 20 mg kg^{-1} DW, and marked on Figure 7 at 17 mg kg^{-1} DW (Reuter *et al.,* 1997; Husted *et al.,* 2009; Schmidt *et al.,* 2013; Schmidt *et al.,* 2016a), unlike the elite cultivars that had Mn concentrations that fell well below this value. As there is no additional Mn added to this hydroponic solution, it is thought that the increase in Mn in the leaf tissue is due to an increase in Mn in the seed and subsequent relocation of the Mn from other parts of the plant. It is also possible that there was recycling of Mn between plants within the hydroponics system, but this is unlikely due to the short time frame of the experiment. Further analysis of the Mn concentration in the seed before germination and the root Mn concentration is needed to identify whether efficient lines have more Mn inherent at germination. Additionally, investigations as to whether an increase Mn concentration in the shoot translates to increased concentration in the seed produced. Both tests could be undertaken using ICP-MS as with the shoot tissue in this study.

These Mn efficient Bere lines also retained almost all their maximum quantum yield of photosynthesis. Other Bere lines, and the landrace Webbs Burton Malting-216, displayed signs of some Mn use efficiency by retaining more of the maximum quantum yield of photosynthesis than the elite lines, but not as much as the highly Mn efficient Bere lines identified, whilst falling below the specified critical deficiency threshold concentration when grown in Mn deficient conditions. The Bere line selected for its reduced efficiency, as identified by the chlorophyll fluorescence, showed no significant difference in Mn leaf concentration from the elite cultivars, displaying that not all Bere lines have high Mn use efficiency. This difference was shown not to be due to a dilution effect, often seen when comparing large and small plants (Jarrell and Beverly, 1981), as the correlation between Mn concentration and shoot biomass was weak and did not have any significant difference. Together these results suggest that there is a range of Mn use efficiency in Bere lines due to adaptation to different environmental pressures found with a range of soil pH within and between the islands. It also indicates that the trait of increased Mn accumulation is not solely responsible for the increases in the Mn use efficiency, highlighting the complexity of pathways with multiple methods of transport (Socha and Guerinot, 2014).

Genotyping and GWAS

Between cultivars there was a large genotypic variation in Mn use efficiency, causing differential Mn^{2+} uptake. A number of studies have been performed to isolate genomic regions associated with increased Mn use efficiency in barley. The first identified plasma membrane-localised metal transport protein capable of transporting Mn^{2+} in barley was *HvIRT1*, located on 4H and 6H when the sequence from Pedas *et al.* (2008) was used in a BLAST search. Two studies have identified loci using RFLP markers from populations crossed with the Mn efficient line Amagi Nijo. The first associated locus, labelled *Mel1*, was identified by Pallotta *et al.* (2000) located on the distal end of chromosome 4HS (Pallotta *et al.,* 2003). The second locus, *Xwg645*, controlling shoot Mn concentration, was found on chromosome 2HL (Lloyd, 2000; McDonald *et al.,* 2001). The locus of most interest in this study was located on 2HL, this corresponds with the *Xwg645* locus identified in Lloyd (2000), further isolating potential QTLs within the locus.

The candidate genes identified in this study had a range of different roles that could contribute to Mn use efficiency and were selected based on:

1) Terpene synthase – produces terpene compounds that act as antioxidants in response to oxidative stress (Rodziewicz *et al.,* 2014), and have been shown to be activated by Mn. Manganese has also been shown to induce ROS production that is corrected with antioxidants (Farzadfar *et al.,* 2016).

2) 3-phosphoglycerate dehydrogenase – found to be associated with the serine biosynthesis in photosynthetic cells (Okamura and Hirai, 2017).

3) Serine/threonine-protein kinase – for their roles in stress signalling (País *et al.,* 2009).

4) MATE efflux family protein – found to be associated with increased Mn uptake in the shoot of Arabidopsis (Rogers and Guerinot, 2002).

5) Yellow stripe-like protein – shown to be involved in increased Mn uptake in Arabidopsis (Waters *et al.,* 2006), rice (Socha and Guerinot, 2014), and thought to be in barley (Zheng *et al.,* 2011).

6) Heavy metal ATPase' – with Cu-transporting shown to be involved in transport of heavy metals such as Mn (Hall and Williams, 2003; Dučić and Polle, 2005) and Cu-ATPase shown to be involved in the transport of other heavy metals into the chloroplast (Seigneurin-Berny *et al.,* 2006).

7) Transporter proteins, for: a) K – shown to play an adverse role in Mn uptake in barley (Alam *et al.,* 2005), b) Zn – identified as a ZnT (found in animal) the plant homologue would be in the CDF transporter family that are associated with metal tolerance (Manara, 2012), and Zn transporters in mammalian cells have been shown to be involved in Mn transport (Kambe, 2012), c) Sulphate – which have been shown to be involved in the transport of heavy metals such as molybdenum (Fitzpatrick *et al.,* 2008) and in abiotic stress response (Gallardo *et al.,* 2014), and d) amino acids – due to the chelation of metals with amino acids that can be transported (Haydon and Cobbett, 2007; Rentsch *et al.,* 2007; Zemanová *et al.,* 2014).

8) Photosystem I P700 chlorophyll a apoprotein – as PSI interacts with PSII, but can also operate independently (Allen, 2002).

9) Aluminium resistance family protein – with Al having been shown to interact with Mn uptake (Wang *et al.,* 2015) with Al-resistance genes conferring tolerance to alkaline soils (Silva *et al.,* 2018).

10) Metal transporter Nramp1 proteins – that are known to be essential for Arabidopsis growth in low Mn conditions (Cailliatte *et al.,* 2010), are similar to Nramp5 that has be shown to mediate Mn uptake in barley (Wu *et al.,* 2016) and rice (Ishimaru *et al.,* 2012). Nramp1 has also been shown to co-operate with ITR1 in iron transport in Arabidopsis (Castaings *et al.,* 2016).

Future Work and Implications

Upon validation of candidate genes and traits, these regions identified could be introgressed into elite cultivars. The goal of this would be to select for the regions of interest identified in this study to transfer them into an elite background, to reduce the negative traits from the Bere line such as low yield and lodging due to its height and weak straw (Martin *et al.,* 2010). This would aid in the focusing and identification of the position of the gene(s) of interest that convey this Mn use efficiency. It would also indicate if there are negative effects or costs associated with the regions of interest. Further validation of the appropriate candidates can be undertaken through methods of positional cloning and genetic transformation to overexpress or suppress the candidate gene (Pflieger *et al.,* 2001; Hu *et al.,* 2008; Aghnoum *et al.,* 2010), allowing further characterisation. The most promising candidates identified in this study for validation would be the metal transporter Nramp1 proteins and the MATE efflux family protein. Selecting for these characterised alleles/genes in the elite population will go towards increasing the Mn use efficiency in the elite cultivars, which this study has shown is limited.

The increase of Mn use efficiency in an elite background without compromising the yield quantity or quality would allow the growth of elite barley in marginal lands, which would not normally economically support the growth of elite cultivars. Further it would provide a buffer to changing environments, preventing deficiencies without the need for routine blanket spraying of Mn foliar fertilizer, thus saving money on purchase and deployment of the chemical. Finally, it will reduce the cases of hidden deficiency that could lead to increased disease (Wilhelm *et al.,* 1988; Marschner *et al.,* 1991; Brennan*,* 1992), increase susceptibility to drought (Hebbern *et al.,* 2009) and salinity (Pandya *et al.,* 2005), and sub optimal use of other minerals such as phosphorus (Allen *et al.,* 2007; Schmidt *et al.,* 2016a). This is due in part to the role of Manganese Superoxide Dismutase, one of the three enzymes for which Mn is essential in barley, that is involved in the response to oxidative stress caused by different abiotic stresses (Szőllősi*,* 2014; Kaouthar *et al.,* 2016; Landi *et al.,* 2017). Together this will help satisfy the increasing demand for food, maintain yields in an increasingly changing climate, and reduce pollution due to chemical runoff.

Evaluating variation in biomass accumulation under salt stress in Scottish barley landrace Bere (*Hordeum vulgare* L.)

Introduction

Salt stress is in the form of sodium (Na) toxicity and salt toxicity, found in sodic and saline soils, respectively. Saline soils are soils that have accumulated salt beyond a critical level, sodic soils are soils with high levels of exchangeable sodium ions and can be accompanied with excess salt (Osman, 2018). The FAO (2015) estimate that 6.5% of land around the world are salt-affected (Figure 10), of which over half are sodic soils. One cause of salinity in soil is the practice of irrigation with brackish waters, causing a steady build-up of salt through evaporation (Umali, 1993; Ayars *et al.,* 1993; Wei *et al.,* 2018). Salt build up in non-irrigated soils is called dryland salinity, occurring through wind dispersal or rising saline groundwater tables leaving deposits of salt that cannot be washed away by leaching and runoff. Dryland salinity can be due to natural fluctuations (primary salinity) or manmade through vegetation clearing (secondary salinity) (Akter, 2017; Pannell and Ewing, 2006; McFarlane *et al.,* 2016). Both of these causes of salinity are likely to increase with manmade climate change due to the increase in need of irrigation and rising sea levels, respectively (Paranychianakis and Chartzoulakis, 2005; Rengasamy, 2006).

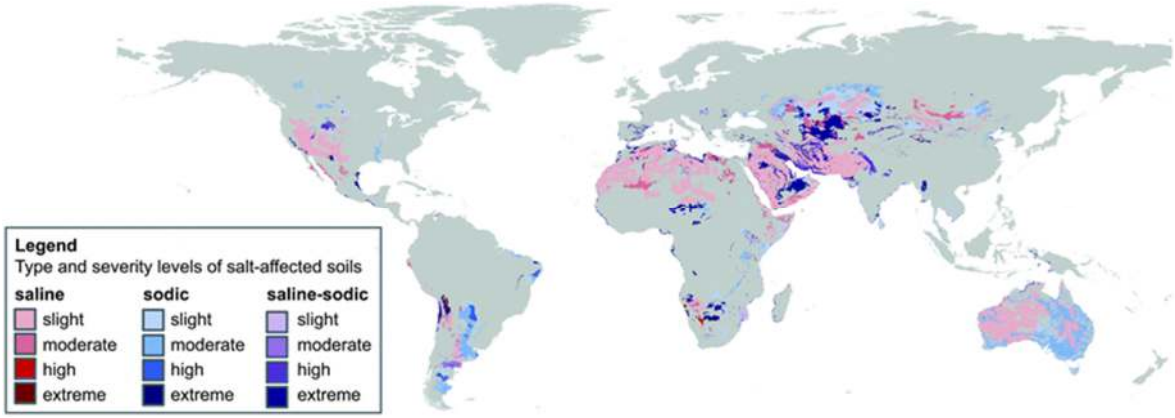


Figure 10) An overview map representing the areas of land that are affected by salt; in terms of saline (red), sodic (blue) and saline-sodic (purple) soils (Wicke *et al.,* 2011).

High levels of soil salinity have a dual negative effect in plants, like most elements there is an ionic effect, but in addition there is an osmotic effect in soils that makes it harder for roots to take up water, simulating aspects of osmotic stresses found due to drought. Osmotic stress occurs when the concentration of salt in the soils reaches a threshold level, usually 40 mM NaCl for most plants, which causes an osmotic pressure on the roots. Though the stress is thought to be mainly due to this osmotic pressure, there is evidence for a non-water potential related effect (Munns and Tester, 2008). This is a rapid effect, occurring within minutes, and results in the decreased growth of new shoots, along with slower emergence of leaves and lateral buds. The ionic toxicity is caused when salt accumulates in the plant tissue to a toxic extent. This is a slower effect, occurring over multiple days, and causes an increased rate of senescence in the older leaves. As ionic stress takes a longer time to manifest, and can only occur at high levels of salinity, osmotic stress has the dominant effect on most plants at most saline levels (Munns, 2002; Munns and Tester, 2008; Roy *et al.,* 2014). Whilst there is some overlap in the plant response, in terms of gene regulation, there is a differential expression of genes between the osmotic and ionic stress (Ueda *et al.,* 2004).

The ionic toxicity plays a role in the interaction with other nutrients due to specific ion toxicity. This is where increased levels of ions, such as Na^+, compete with essential nutrients for uptake and metabolism in the plant, potentially causing a deficiency in nutrients such as P, N, Ca, and K (Parihar *et al.,* 2015). The latter (K) in particular has shared transporters with Na ions such as the AKT1 (aka RAC-alpha serine/threonine-protein kinase) and HKT1 (High-affinity Potassium Transporter 1) co-transporters that use the high K^+/Na^+ ratio, maintained during ordinary physiological conditions, to transport both ions (Blumwald *et al.,* 2000). Saline and sodic soils are also associated with limited micronutrient solubility, resulting in an interaction that increases the deficiency of micronutrients such as Cu, Fe, Zn, Mo and Mn (Grattan and Grieve, 1998). The latter (Mn) has been shown in barley causing a reduction in photosynthesis, and thus yield, but can be corrected by foliar spray of Mn (Cramer and Nowak, 1992; Pandya *et al.,* 2005). Boron toxicity is also a concern in saline soils as salinity has been shown to have a negative interaction with boron tolerance, thus increasing the sensitivity to boron toxicity in numerous species including wheat (Wimmer *et al.,* 2003).

Due to the different types of stresses produced by saline conditions, there are different mechanisms of resistance. The three broad categories of resistance are: the exclusion of Na ions from the leaf tissue to prevent ionic stress, the tolerance of osmotic stress, and the tolerance of ionic stress, such as Na ions build-up in the leaf tissue, through methods such as compartmentalisation (Munns and Tester, 2008). An example of the latter in barley is from the Widodo *et al.* (2009) study on the salt tolerant barley cultivar Sahara that showed high levels of salt concentrations in the leaf tissue without apparent damage. This lack of damage was suggested to be due to the ability of the plant to sequester Na ions into the vacuole, maintaining the K^+:Na^+ ratio in the cytoplasm. The other broad category mentioned to deal with ionic stress is an avoidance mechanism using ion exclusion, where ion transporters actively efflux sodium ions from the root tissue before they can diffuse into the xylem - High-affinity Potassium Transporters (HKTs) are thought to be an important gene family in this process. An example of this in action is shown in Figure 11 in which wild type barley with diminished biomass on the left, and the healthy transgenic barley encoding for a vacuolar proton pump on the right, when grown in a field site with saline soil (Schilling *et al.,* 2014). The last resistance mechanism category is the poorly understood category of osmotic tolerance (Munns *et al.,* 2006; Munns and Tester, 2008; Roy *et al.,* 2014). This mechanism is often associated with drought tolerance, an example of this is the *ari-e* dwarfing gene found in Golden Promise that conveys a greater water use efficiency accounting for tolerance to salt stress and implies a tolerance to drought (Forster, 2001). Additionally Widodo *et al.* (2009) also suggested that the increased salt tolerance noted in their study could be due to increased metabolite production to cope with increased osmotic potential.

Figure 11) Overhead view of transgenic barley (**right**) that had been encoded to produce a vascular proton pump to successfully alleviate salt stress, compared to the wild type (**left**) that exhibits diminished biomass growth due to salt stress (Schilling *et al.,* 2014).

Due to lack of economically practical screening methods, and the fact that salinity tolerance is a highly complex trait composed of resistance to both ionic and osmotic stress, conventional breeding in barley is limited. Additional problems arise in the differential result, and possibly mechanism, of resistance between salt tolerance in seedling and germination, and between hydroponic and soil systems. Recent advancements in genotyping have allowed for the identification of QTLs and the use of marker assisted breeding (Mano and Takeda*,* 1997; Tavakkoli *et al.,* 2010; Zhou *et al.,* 2012a; Ashraf and Foolad*,* 2013). These tools have the potential to identify regions associated with salinity tolerance from sources such as tolerant landraces (Newton *et al.,* 2010; Allel *et al.,* 2016; Dwivedi *et al.,* 2016). One identified tolerance mechanism in barley that could be used in breeding programs are HKTs (Hamamoto *et al.,* 2015). In particular HvHKT2;1 from barley that causes an increased sodium ion uptake, but with an associated increase in Na^+ translocation that correlates with an increased tolerance to salt (Mian *et al.,* 2011). Alternatively, HvHKT1;5 has shown differential expression between tissues in salt tolerant lines. The salt tolerant lines show increased expression of HvHKT1;5 in the roots and a decrease in expression in the leaf sheaths compared to the salt sensitive lines when exposed to salt. This causes an increased exclusion of sodium ions from the roots, and reduction of Na transport to the leaf tissue (Hazzouri *et al.,* 2018). Additionally, proteome studies between a salt tolerant and sensitive barley line by Mostek *et al.* (2015) have shown a differential expression of proteins involved in a number of different functions such as signal transduction, detoxification, protein folding processes, and cell wall metabolism. The latter of which has often been associated with abiotic stress response, with one main response including cell wall thickening (Le Gall *et al.,* 2015), and differential composition has been shown to influence the passage of sodium ions (Byrt *et al.,* 2018).

The aims of this study were to identify Bere and other landrace lines that are able to maintain biomass when grown in saline and sodic growth media. This data was used along with the genotypic data to identify genomic regions associated with this trait using a GWAS. This region can then be searched for encoded candidate genes that are speculated to have a putative function associated with salt tolerance. The goal of this project would be to identify candidate genes for future characterisation and possible incorporation into commercial breeding programs to breed for salt tolerant barley crops.

Results

Landrace Screen

1.1.1.7. Raw Data Analysis

When comparing the data of the different sub-categories for each of the variates – dry weight, fresh weight, tiller number, and height – the difference in salt concentrations and sub-category type was significant ($p<0.001$), with the exception of the sub-category difference in dry weight ($p=0.056$). No significant difference for the interaction of these treatments was seen for any variate (p-values=0.051-0.660). The elite cultivars produced the most fresh weight, with an average of 80 g, with the Bere lines producing the least at only 67 g on average. No height difference was seen between the Beres and elites at approximately 71 cm, but the other landraces were significantly different at 65 cm. No tiller differences between landraces and elites were seen with approximately 12.3, but the Beres were significantly different with 9.6.

Individual analysis of the same varieties shows similar results. Highly significant differences are seen in all variates between differing salt concentrations and between lines/cultivars ($p<0.001$). It is also seen that there is no significant difference for the interaction between the sub-category type and the salt concentration (p-values 0.206-0.587).

1.1.1.8. Fitted Line Data Analysis

Using the slope integer from the fitted linear equation there was a significant difference between the three sub-categories for dry weight ($p=0.028$) and tiller number ($p=0.007$), but not for fresh weight ($p=0.239$) and height ($p=0.127$). As a percentage of the control, this significance in dry weight and tiller number is not preserved, though in the former there was a trend towards significance ($p=0.051$), and became significant for height ($p=0.020$). The loss in dry weight with increased salt (Figure 12) in the elite cultivars is approximately double that of the Bere lines at 0.07 g per mmol/kg of NaCl (or 0.39%) compared to 0.04 g per mmol/kg of NaCl (or 0.17%).

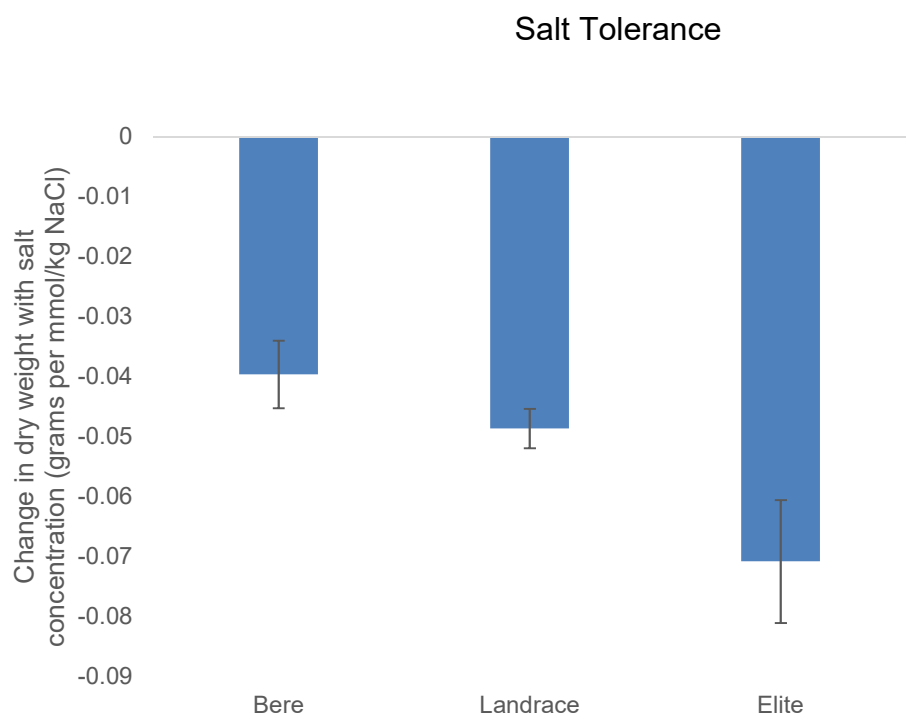


Figure 12) The average change in dry weight with increasing salt concentration for 146 lines/cultivars of barley divided into three groups, Bere, Landraces and elites (n= 37, n=104, and n=5,respectively). Error bars represent the standard errors in positive and negative directions.

When the slope integer data for the individual lines/cultivars was compared, a significant difference was found between dry weight ($p<0.001$) and fresh weight ($p=0.011$), and when comparing the slope data calculated as a percentage of the control (p-values =0.040 and 0.010, respectively). Height and tiller number were not significantly different. Seventeen lines had slope integers that showed an increase in dry weight with increased salt levels (Figure 13), four of these were Bere lines. Three of these lines showed significantly positive levels: Prize Prolific-196, Bere-118, and Bere 49 A 27 Shetland, with increases of 0.043, 0.040, and 0.032 g per mmol/kg, respectively (and increases of 0.35, 0.45, and 0.4% per mmol/kg, respectively). Swannek-213 had a small increase of 0.016 g per mmol/kg, but due to its small nature was 0.98% per mmol/kg. Fifteen lines had large weight reductions of 0.1 g per mmol/kg (and over 0.5% per mmol/kg), including elite cultivar Waggon and Bere 39 A 16 Berneray. The remainder were other landraces, with Skadu Local "Oldings", and Old Irish-184, showing the largest decreases of 0.169, 0.157, and 0.149 g per mmol/kg, respectively (0.57, 0.65, and 0.85% per mmol/kg, respectively).

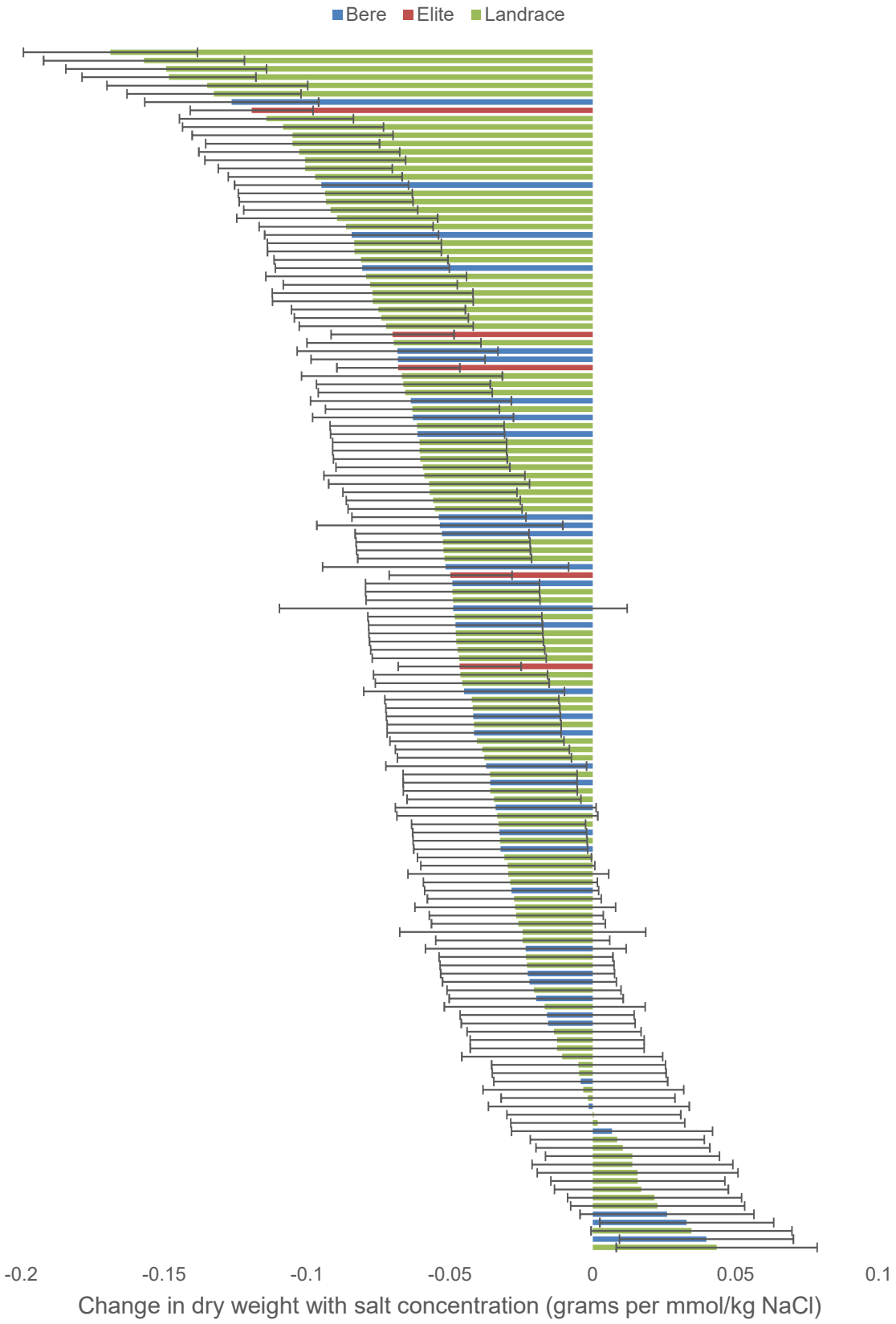


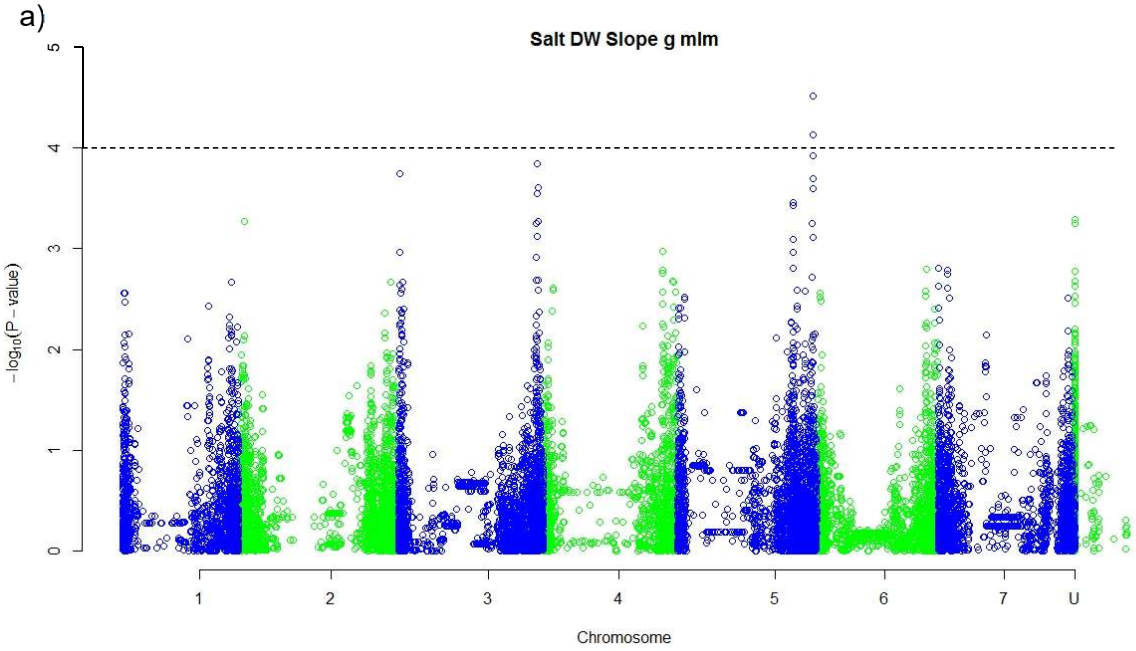
Figure 13) The average change in dry weight with increasing salt concentration for 146 lines/cultivars of barley grown for approximately 70 days in universal compost. Error bars represent the standard errors in positive and negative directions.

1.1.1.9. Genome-Wide Association Study (GWAS) Analysis

From the 37,242 markers used, 10,593 were removed as having low (<10%) minor allele frequency and a further 30 because of a low call rate. Of the 140 lines used, 13 were excluded because their heterozygosity was too high and nine due to being identical by state (IBS).

The QQ plots showed that the MLM approach for the mean fluorescence score when grown in 0 µM Mn had the smallest deviation from the expected null distribution, but with little difference from the EG model. The Manhattan MLM plot for both the slope integer data (Figure 14; weight (a) and percentage (b)) identified one region of significance on the distal end of chromosome 5HL. Only six markers with p-values of <0.001 in both analyses were identified (others were identified in only one analysis), four of these were the only markers with p-values of <0.0001. One was found at the distal end of 3HL, the other five were found in the distal end of 5HL, three at 651.49-651.52 Mb and two at 651.20 Mb. The five markers identified in 5HL were amongst the largest negative effects, with decreases of 0.023-0.028 g per mmol/kg and 0.155-0.185% per mmol/kg. The marker identified on 3HL was shown to have the 2nd/3rd highest positive effect, with an increase of 0.029 g per mmol/kg and 0.169% per mmol/kg.

a)



b)

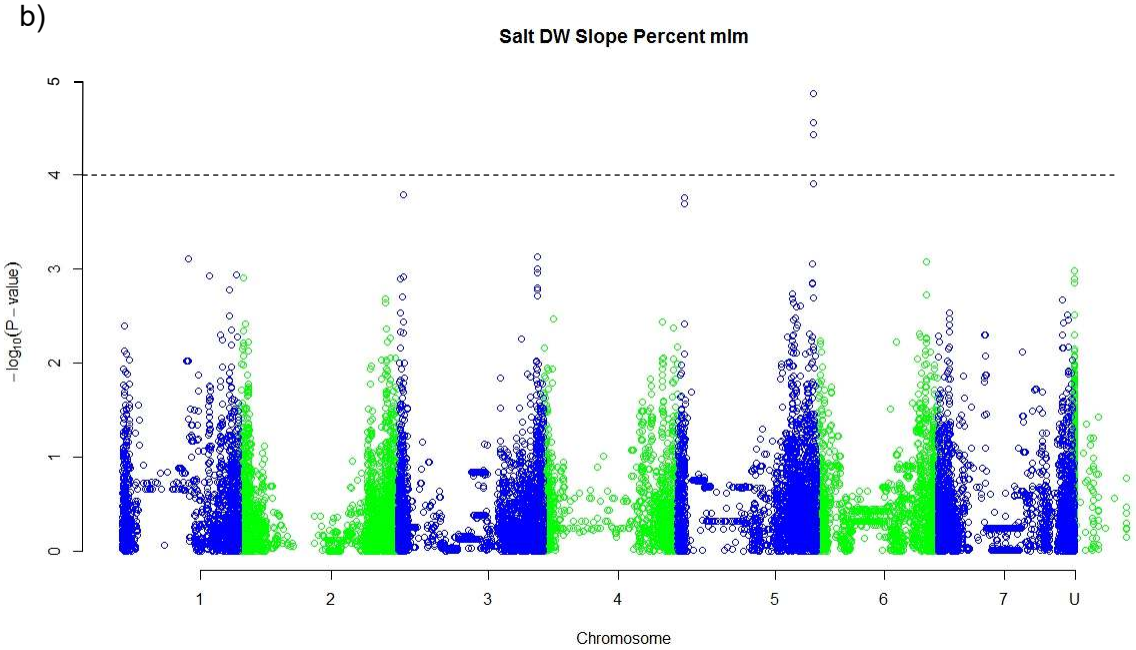


Figure 14) A Manhattan plot of a Genome-Wide Association Study undertaken using a Mixed Linear Model on the average change in dry weight with increasing salt concentration as **a)** weight, **b)** percentage of the average control weight; data generated using an ANOVA. Depressions in marker significance observed in the centre of each chromosome are due to reduced marker density around the centromere of the physical map.

Within the region 651.10-651.60 Mb of 5HL there were a total of 29 associated genes, of which four were identified as candidate genes (listed in Table 5) that encode for: a Lysine-specific demethylase REF6, an Actin 7, a Ferredoxin 3, and an Acyl-CoA-binding domain-containing protein 4, the latter of which contains the two most significant markers for data sets, but the former of which is a low confidence gene. The marker at 670.25 Mb on 3HL is positioned with a gene encoding for an Amino-acid permease BAT1 homolog (Table 5), no other candidates were identified around this marker.

Table 5) Candidate genes identified in relation to salt tolerance in regards to biomass growth, with the chromosome and position on the physical map listed.

Gene Name	Chr	Position	Annotation
HORVU3Hr1G105920.6	3HL	670.25	Amino-acid permease BAT1 homolog
HORVU5Hr1G117860.5	5HL	651.33	Lysine-specific demethylase REF6
HORVU5Hr1G117900.1	5HL	651.48	actin 7
HORVU5Hr1G117910.3	5HL	651.49	ferredoxin 3
HORVU5Hr1G117970.2	5HL	651.52	Acyl-CoA-binding domain-containing protein 4

Of these, actin is the most common annotation representing 0.23% of the 73586 genes listed in BARLEX the barley genome explorer (Colmsee *et al.,* 2015), at 172 genes genome-wide. The remaining annotations – Lysine-specific demethylases, ferredoxins, Acyl-CoA-binding proteins, and Amino-acid permeases – are less common representing ≤0.08% of the genome each, at 59, 30, 23, and 5 genes, respectively.

Discussion

The problem of salt toxicity is limited to localised regions, though affects a large area, 6.5% of lands worldwide (FAO, 2015), and is becoming an increasing problem with the irrigation of land with brackish water (Umali, 1993; Ayars *et al.,* 1993; Wei *et al.,* 2018) and increasing dryland salinity due to climate change (Rengasamy, 2006) and deforestation in temperate zones (Sahagian, 2000). There is need to increase production on more marginal lands where it is already a problem and to preserve yields on lands that are being degraded by increasing salt concentrations. One method of elevating yield on these lands would be to increase the tolerance of the crops to salinity through breeding (Munns *et al.,* 2006). For this to be successful salt tolerance genes need to be identified, and a viable source of these genes could be from landraces that grow in marginal soils that contain elevated salt levels. This study has assessed landrace lines for their ability to maintain biomass, and other indicators, in saline conditions. This allowed for the identification of differences between lines, which follow overarching differences between sub-categories, as well as genomic regions associated with the maintenance of biomass in saline conditions, along with a number of genes with putative functions associated with salinity tolerance.

Effect on Biomass

A screening of the landrace collection showed that there were no differences in the way that the two treatments interacted with each other. However, when this data was fitted to a linear model to see how the different weights, height, and tiller number changed with increasing salt concentration it could be seen that there was a significant difference between the dry weight when comparing both sub-categories and individual lines/cultivars. This revealed that the elite cultivars lose approximately twice as much dry biomass with increasing salt concentrations as the landrace lines, at a loss of 0.39% per mmol/kg, showing that the elite cultivars are less tolerant to salt concentrations in the compost. This is comparable to the effect of salt on dry weight from Long *et al.* (2013) that showed an average (of 192 genotypes) decrease in shoot dry weight of 67% from 0-200 mM NaCl (equivalent to 0-200 mmol/kg) in a hydroponic system, or 0.34% per mM. In this study the most salt tolerant elite genotype lost 48% over the same range, or 0.24% per mM, which is more than the average of the Bere lines at

0.17%. However, it is possible that there was decreased levels of salt in the compost of this study due to decreased salt concentrations through leaching. A similar experiment using gravel with nutrient solution with increasing salt concentrations from Rawson *et al.* (1988) showed similar levels of decrease, with the most tolerant barley line showing a 38% average loss in salt conditions (averaged 175-250 mM), or 0.18% per mM (Munns *et al.,* 1995). When these results are shown individually it can be seen that the spread of the landrace lines, both Bere and non-Bere, is large, with the elite cultivars all above average. From these it was possible to identify a number of Bere and non-Bere landraces that have no or positive changes in dry weight with increasing salt levels, suggesting that they are very salt tolerant. This positive change in dry weight could be due to the effect of salt concentrations on the availability of nutrients (Grattan and Grieve*,* 1992), providing a nutrient profile to which the lines are more adapted to. It is also possible to find Bere and non-Bere landraces that have very large negative changes, equal to the elite cultivars most affected by salt – suggesting that the tolerance is not a uniform trait across all landraces.

Genotyping and GWAS

The GWAS undertaken in this investigation identified one significant QTL of interest at 5HL, and another possible peak with strong markers at 3HL. Within the region at 5HL there are a number of genes encoding for proteins of interest such as a) Lysine-specific demethylase REF6 – selected as it has a histone demethylase domain and over expression of a histone demethylase gene in Arabidopsis has been shown to improve salt tolerance (Shen *et al.,* 2014); b) Actin 7 – selected as salt stress has been shown to affect actin filament assembly and has shown to be necessary in salt tolerance in Arabidopsis (Wang *et al.,* 2011); c) Ferredoxin 3 – selected as salt stress has been associated with an increase in ferredoxin-dependent glutamate synthase activity (Berteli *et al.,* 1995) and ferredoxin-thioredoxin-reductase (Zhou *et al.,* 2009); and d) Acyl-CoA-binding domain-containing protein 4 – selected as Acyl-CoA-binding proteins have been shown to interact with other proteins in response to abiotic stresses (Raboanatahiry *et al.,* 2015), with overexpression in Arabidopsis shown to improve drought tolerance (Du *et al.,* 2016). One candidate gene was identified around the markers on 3HL, encoding for an Amino-acid permease BAT1 homolog – selected as Amino-acid permeases have

been shown to increase proline accumulation under salt stress conditions (Wang *et al.,* 2017). As mentioned previously, HKT genes are the most recognised genes for salt tolerance (Hamamoto *et al.,* 2015; Hazzouri *et al.,* 2018), however none were identified in the regions identified in this study, indicating different mechanisms like those listed above through either tissue tolerance or different methods of exclusion (Munns, 2009).

Future Work and Implications

Further testing of the identified lines should be undertaken to gain a greater understanding into the nature and extent of the salt tolerance. This could include assessing the root growth in compost or soil of differing salinities, assessing the Na concentrations within the leaves grown in these concentrations, testing the biomass of selected lines in a more complete range of salt concentrations, and assessing the yield when grown in these concentrations. These lines, such as Prize Prolific-196, Bere-118, and Bere 49 A 27 Shetland, could be used directly for soils that are highly salt affected such as coastal soils and areas irrigated with poor quality water. Additionally, further testing on these lines with ratios of differing salts is necessary as whilst sodium salts are the most common, salts such as chlorides and sulphates of calcium and magnesium are also found in soils (Abrol *et al.,* 1988). This is particularly necessary as the ratio of sodium salt to other salts is much lower in costal saline soils that are sea influenced (Mugai, 2004), such as some Scottish islands where Bere barley is grown (Dry and Robertson, 1982; Dry, 2016). This will allow us to determine if the improved growth is a tolerance to saline or sodic soils, or a combination of both.

Once a more detailed understanding of the salt tolerance has been attained, the regions that have been identified in the biomass growth study can then be introgressed into elite cultivars. Introgressed lines would help further isolate the gene(s) associated with the identified resistance, that could then be bred into an elite background. The benefit of having an elite line with additional tolerance to salt conditions would include the ability to grow elite lines on more marginal land, and to increase the robustness of the elite crops to seen and unseen salination e.g. floods, or irrigation with brackish waters to maintain and increase production (Ismail and Horie, 2017). Salt stress is also highly related to drought stress, both having similar or identical effect on water deficiency and osmotic effect (Hu

and Schmidhalter, 2005; Katerji *et al.,* 2009), thus the identified mechanisms that offer an increase in salt tolerance should be assessed for drought stress tolerance.

Identification and introgression of regions that are able to alleviate both saline and drought stress would be of importance particularly in poorer regions as the most common reason for the necessity of watering with brackish water is the prevalence of drought (Hillel, 2000). This is of increased importance as areas of salinity and drought are expected to increase over the upcoming years (Wang *et al.,* 2003), exacerbated by climate change causing unpredictable weather and rising water tables (Munns and Gilliham, 2015). Additionally, increased resistance to saline conditions allows for the use brackish water to alleviate water stress during periods of extreme drought in areas relying on rain water as the primary water source such as the Mediterranean (Hamdy *et al.,* 2005).

Evaluating the infection variation of the foliar disease 'Scald' (*Rhynchosporium commune*) in the Scottish barley landrace Bere (*Hordeum vulgare* L.)

Introduction

The *Rhynchosporium* genus comprises of haploid hemibiotrophic fungi that infect grasses such as rye, triticale, and barley. Originally the pathogens of these three crops were classified as pathotypes of *R. secalis*. A study by Zaffarano *et al.* (2011) identified that the pathotype infecting barley was a separate species, and it was subsequently labelled as *Rhynchosporium commune*. This host adaptation is mediated through effector proteins that stall the development of the fungi *in planta* in order to extend the biotrophic phase (Clark *et al.,* 2008; Penselin *et al.,* 2016). The disease is found in all areas where barley is grown but is especially prevalent in areas of cooler climate. Yield losses have been reported ranging from 10% to 45%, and economic yield is reduced due to inferior quality grain for products such as malting barley. In the UK this equates to an estimated £7.2 million worth of losses in barley after fungicide treatments (Avrova and Knogge, 2012; Paveley *et al.,* 2016).

Whilst the fungus can be seed-borne, the majority of infection comes from the spread of the fungus originating on crop debris of the previous season, or from volunteer plants that host the fungus over winter. This spread is usually via rain splash which causes dispersal of spores to new hosts, air-borne transfer of spores may also transmit the pathogen but is thought to be much more limited. Seed-borne infection is thought to remain symptomless *in planta* for several months (Clark *et al.,* 2008; Fountaine *et al.,* 2010; Avrova and Knogge, 2012). Infection via the more common rain splash dispersal can also remain symptomless as the biotic phase does not produce the characteristic symptoms of scald shown (Figure 15) (Zhan *et al.,* 2008). The typical symptoms signal the necrotic phase of leaf scald and start with a paling of an oval-shaped lesion, later surrounded by a necrotic ring of tissue often with leaf yellowing around it. The lesions look like scald marks and can coalesce into larger regions causing general necrosis and potential death of the leaf. Symptoms usually occur on the leaves, leaf sheaths and ears (Clark *et al.,* 2008; Avrova and Knogge, 2012). The current strategies to reduce the

number of rhynchosporium outbreaks include: agronomic practices such as crop rotations, cultivar mixtures, and fungicide use, as well as genetic resistance through breeding. However, due to rapid adaptation of the pathogen populations, individual treatments are often inadequate by themselves (Zhan *et al.,* 2008). This rapid adaptation of pathotypes – in response to changing environments, resistant cultivars, and fungicide treatments – is thought to be caused, in part, by the sexual recombination of the pathogens, which is identified to have occurred in all field populations (McDonald, 2015). This causes selective pressures that account for approximately three quarters of the global *R. commune* species diversity being found in the field (McDonald, 2015) and an increasing race complexity with time (Zhan *et al.,* 2012). Additionally, the prohibition of fungicides due to safety and political reasons exhibit a flaw in the reliance on chemical treatment as the sole form of control. An example of this is the European Union's imminent ban of the most commonly used fungicide in the USA and UK – chlorothalonil (Hillocks, 2012; European Commission, 2019). Chlorothalonil is a broad-spectrum fungicide used to control a range of fungal pathogens including *R. commune*, but has been reported to cause a reduction in biodiversity and a change in ecosystem function (McMahon *et al.,* 2012; Creissen *et al.,* 2016). Together these shows a need for the development of ever more genetic resistance to *R. commune*.

Figure 15) Images showing the characteristic scald pattern of infection of barley with *Rhynchosporium commune*. Sourced from Hofmann (2014).

Resistance genes against *R. commune* infection previously had different naming methods with allelic differences and duplicates having different unrelated names. Work to make the nomenclature unified

was undertaken by Bjørnstad *et al.* (2002), assigning the Rrs prefix to almost all, consolidating down to: Rrs1 with 11 alleles, Rrs2 with two, Rrs3 with one, Rrs4 with two, Rrs12-14 each with one, and four unconfirmed R genes. Other resistance genes against *R. commune* that were not mentioned or that have been since identified include: Rrs5, rrs6-8, Rrs9, Rrs10, rrs11 (Takeuchi and Fukuyama, 2009), Rrs15 (Genger *et al.,* 2005), Rrs16 (Pickering *et al.,* 2006), a second Rrs15 found in a different location (Wagner *et al.,* 2008) which has been suggested to be changed to Rrs17 by Zhan *et al.* (2008), and the latest gene labelled to date, Rrs18, found on 6HS by Hofmann (2014) and confirmed by Coulter *et al.* (2018) (Figure 16). A number of these resistance genes originate from landraces (Bjørnstad *et al.,* 2004; Hofmann *et al.,* 2013; Hanemann *et al.,* 2010). It has been suggested that Scandinavian and other northern European landraces might be a prime source for resistance to rhynchosporium as it is speculated that *R. commune* originated in this area providing diverse *R. commune* populations and the longest period of landrace selection for rhynchosporium resistance (McDonald, 2015), suggesting Bere barley is a suitable candidate. Other sources of resistance could come from the alternate, broad-based, PAMP triggered immunity (PTI). An example on how this could be used is the identification of a *R. commune* PAMP by Franco-Orozco *et al.* (2017) that induces PTI in solanaceous species, but not monocots. By replicating this pattern recognition receptor (PRR), through identification or induction of mutations, a new resistance mechanism may be transferred between plant families. Study of landrace material may provide a resource of novel PRRs that could offer broad-based resistance.

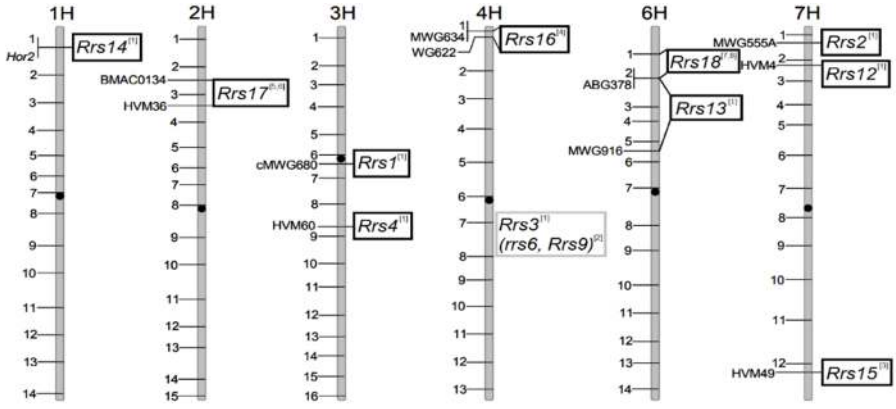


Figure 16) The location, or approximate location for Rrs3, of mapped a selection of resistance genes for *Rhynchosporium commune* on the barley bin map, with associated markers. Chromosome 5H is not depicted as there are no associated markers at present. The references are as follows; 1) Bjørnstad *et al.* (2002), 2) Takeuchi and Fukuyama (2009), 3) Genger *et al.* (2005), 4) Pickering *et al.* (2006), 5) Wagner *et al.* (2008), 6) Zhan *et al.* (2008), 7) Hofmann (2014), and 8) Coulter *et al.* (2018). Figure adapted from Zhan *et al.* (2008) and Hanemann (2009).

The aims of this study were to identify if the Bere lines have an inherent resistance to rhynchosporium as suggested by anecdotal evidence (Mahon *et al.,* 2016), as well as identifying individual lines that have high resistance. To then use this data to identify genomic regions associated with this trait, and any candidate genes in this region.

Results

Controlled Landrace Screen

1.1.1.10. Sub-category Analysis

There were significant differences in infection lesion area between the sub-categories ($p<0.001$), isolates ($p<0.001$), and interaction of these treatments ($p=0.043$). There were significant differences also in lesion severity between the: isolates ($p<0.001$) and the isolate sub-category interaction ($p=0.048$), but not between sub-categories ($p=0.066$). Comparison of the lesion area (Figure 17a) indicated that lesions in Bere lines are smaller. Lesions from isolates 13-13, L73A, and L77 caused smaller lesions in Beres than the other landrace lines, the latter two isolates in Beres also caused smaller lesions compared with the elite cultivars. Isolate L2A showed no significant difference between sub-categories. The isolates also showed differences between each other in size in all sub-categories, with isolates L73A, L77, 13-13, and L2A causing average lesions of 33, 30, 19, and 13 mm^2, respectively. Comparison of lesion severity (Figure 17b) showed the opposite trend to that of the area, with lesions in Bere lines showing greater lesion severity. Lesion severity from isolates 13-13 and L2A, that caused minimal differences in the lesion area, showed greater lesion severity in the Bere lines than both elite cultivars and other landraces. The latter also showed less lesion severity in the elite cultivars compared to the other landrace lines. Conversely, isolate L77 caused greater lesion severity in the other landrace lines than in elite cultivars and Bere lines. Isolate L73A showed no differential interaction with the sub-categories.

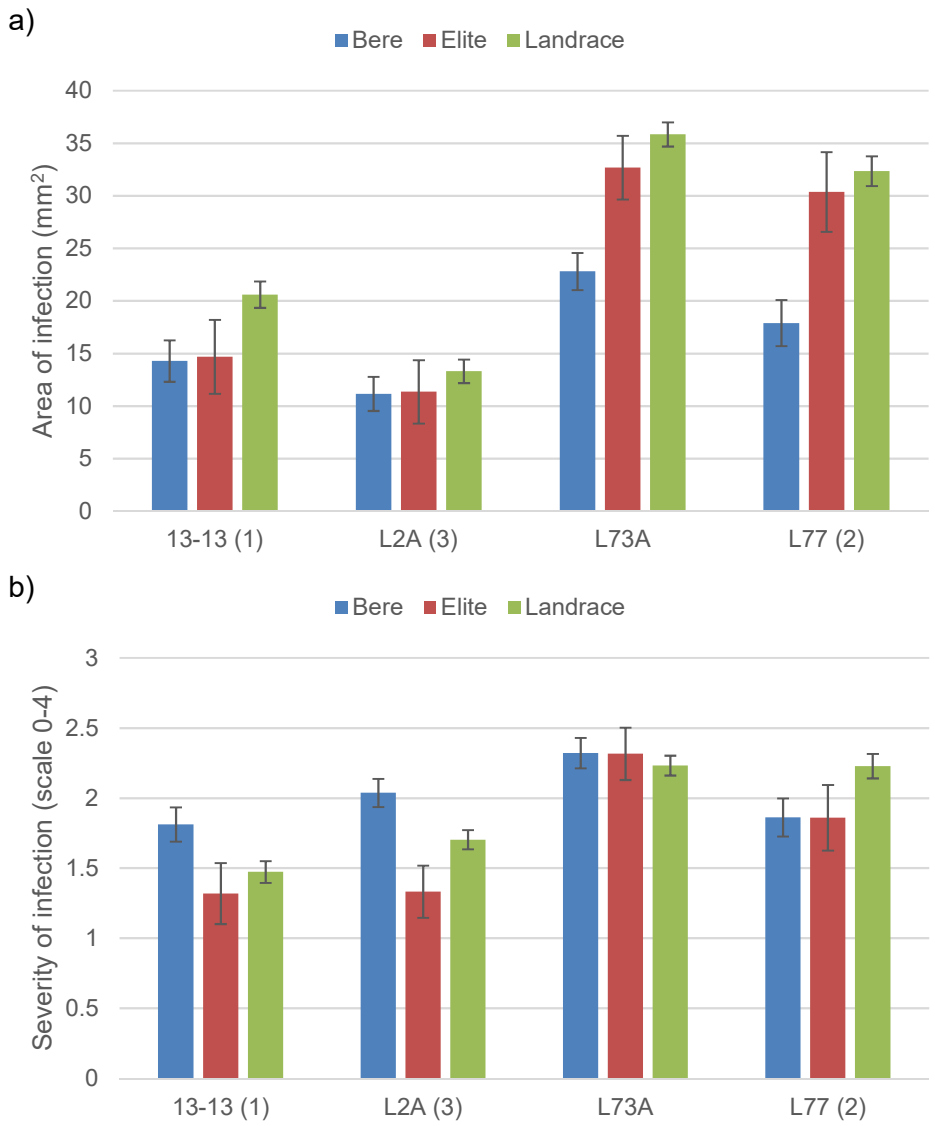


Figure 17) Comparison of the *R. commune* infection for 132 lines/cultivars of barley divided into three, unequal, sub-divisions (35, 87, and 10 for the Bere, other landraces, and elites respectively) with four different isolates, looking at **a)** area of infection, and **b)** lesion severity of infection. Error bars represent the standard errors.

1.1.1.11. Individual Line/Cultivar Analysis

Individual analysis also shows significant differences between the: lines/cultivars, isolates, and interaction of these treatments ($p<0.001$), for both lesion area and lesion severity. Mean isolate data for the lesion area (Figure 18a) shows a spread of each sub-category. Most Bere lines are amongst the smaller lesions, with nine of the ten smallest being Beres. The elite cultivars similarly show a spread of lesion sizes, with two being of similar size to the smallest of the Beres. Mean lesion severity data (Figure 18b) similarly shows the majority of the Bere lines with high lesion severity, with the highest three being Beres. However, the line/cultivar with the lowest lesion severity is also a Bere line.

Elite cultivars show most lesions having low lesion severity, many comparable to the lowest Bere line, but elite cultivar Concerto has high lesion severity comparable to the highest Bere lines.

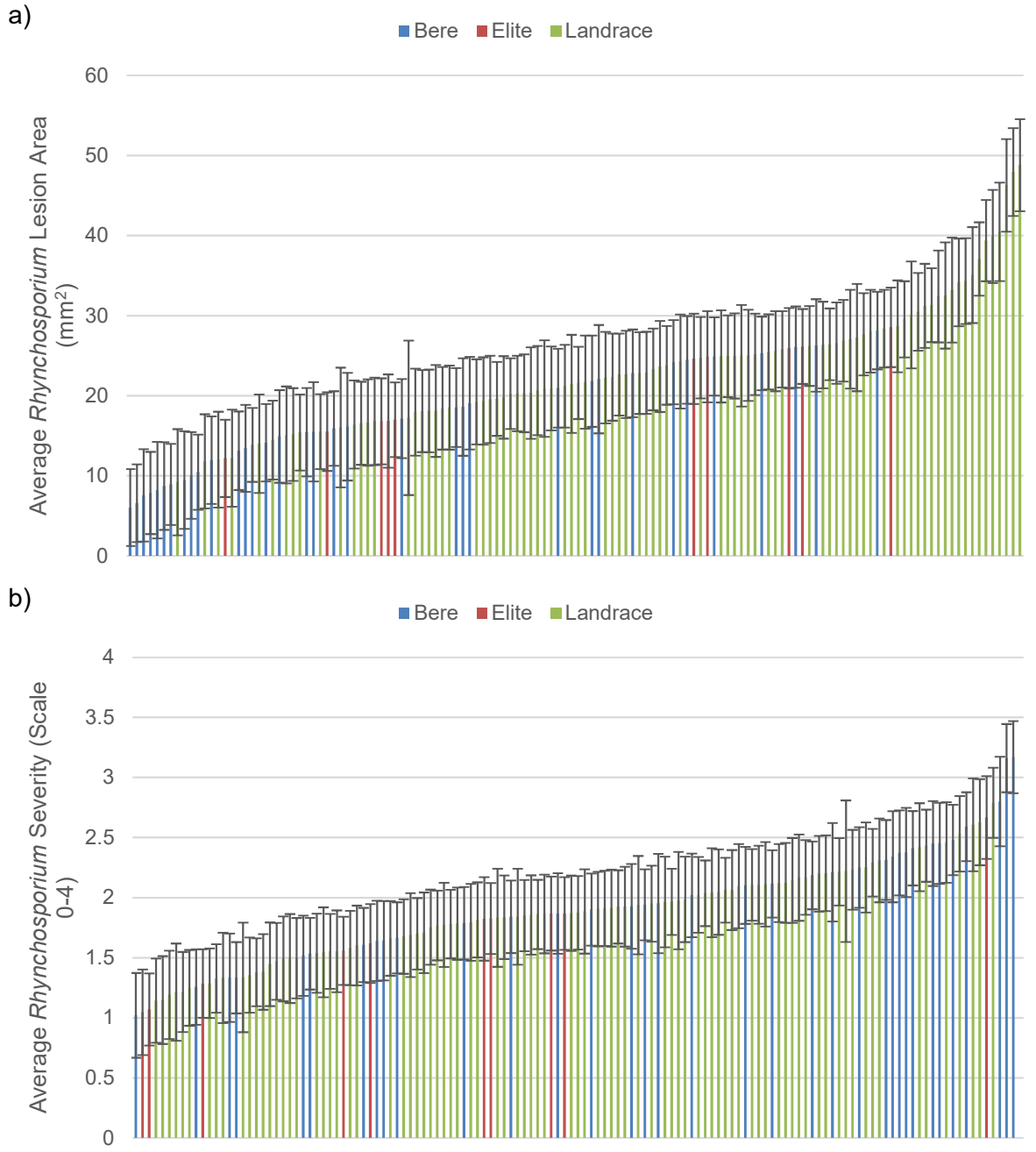


Figure 18) Comparison of rhynchosporium symptoms for 132 lines/cultivars of barley, averaging the four different isolates, looking at **a)** area of infection, and **b)** lesion severity of infection. Error bars represent the standard errors.

Comparison between lesion size and lesion severity, for both the averaged data of all isolates (Figure 19) and within the individual isolates, show no correlation. Likewise, no correlation was found comparing all the individual isolates against each other for both lesion area and lesion severity.

From the comparison between separate isolates, individuals with consistently small lesion size and lesion severity can be identified. Bere 55 A 33 has the smallest lesion size of all the Beres with all four isolates, amongst the ten smallest lines/cultivars with three isolates. Other Bere lines include Bere 45 A 23, Bere 58 A 36 Eday, and Bere 8-125, all produce no lesion with L77 and have small lesions with another two isolates, but have average sized lesions with isolate 13-13. The lines/cultivars with the largest average lesion size were all non-Bere landraces (Figure 18a), but only two – Spratt Archer 37/6/3-205 and Irish Goldthorpe-222 – had above average lesion sizes in all isolates. Kenia-M08, Binder-M08, and Scotch Common-M08 had amongst the largest lesions, but had the smallest lesions with isolate L2A.

The Bere line with the smallest overall lesions, Bere 55 A 33, has amongst the greatest lesion severity, fitting the pattern of inverse lesion size and lesion severity seen in the sub-category analysis. However, this pattern is not seen in the other lines with small lesion sizes as Bere 45 A 23, Bere 58 A 36 Eday, and Bere 8-125 all have amongst the least lesion severity. The two elite cultivars with the least lesion severity (Figure 18b) are KWS Irina and Westminster. Of the lines identified above with large lesions, nearly all also had high average lesion severity, the exception to this was Scotch Common-M08 but that did have high lesion severity with half the isolates.

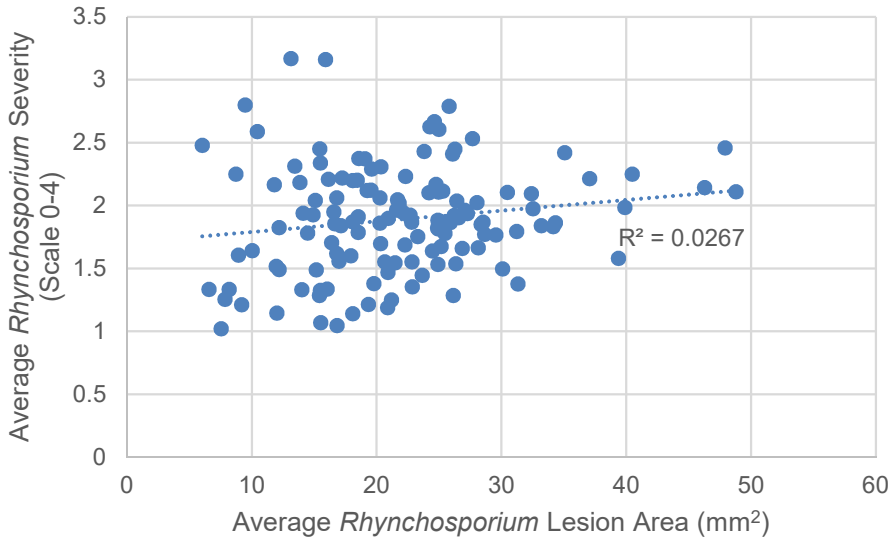


Figure 19) Correlation between the average rhynchosporium lesion size vs the lesion severity in 132 lines/cultivars compared with the mean of all four isolates. For the correlation the line of best fit along with the coefficient of determination (R^2) value is given, p=0.061.

1.1.1.12. Genome-Wide Association Study (GWAS) Analysis

As some lines had no viable leaves for assessment with certain isolates the number of excluded markers and lines for each analysis differed. The number of markers removed, from the 37242 total, for having low minor allele frequency and a low call rate are recorded in Table 6. The total number of lines and those excluded because their heterozygosity was too high and being identical by state are recorded in Table 7.

Table 6) Number of markers excluded in the GWAS quality control on data collected for individual *R. commune* isolates due to having low (<10%) minor allele frequency and/or a low call rate, excluded from the 37242 total, plus the number of markers remaining for the analysis.

Isolate	Low minor allele frequency	Low call rate	Remaining Markers
13-13	10536	43	26672
L73A	10406	41	26804
L77	10799	51	26403
L2A & Average	10680	43	26529

Table 7) Number of lines used in the GWAS quality control on data collected for individual *R. commune* isolates plus the number excluded due to having high heterozygosity and/or being identical by state (IBS), plus the number of lines remaining for the analysis.

Isolate	Total Lines	High heterozygosity	IBS	Remaining Lines
13-13	120	12	8	100
L73A	120	12	8	100
L77	114	12	8	94
L2A & Average	122	12	9	101

The QQ plots show that a Mixed Linear Model approach had the smallest deviation from the expected null distribution for the averaged isolate and isolate L73A data sets, for both lesion area and lesion severity, and the L77 isolate lesion area data. Likewise, for isolates 13-13 and L2A an EIGENSTRAT approach had the lowest deviation for both lesion area and lesion severity, and the isolate L77 lesion severity data.

The Manhattan plots for the mean isolate data (Figure 20) showed no peaks above the threshold of $-\log_{10}(\text{p-value}) \geq 4$, with the only areas showing indications of regions of interest being at 2HL for lesion area (a) and 5HL for infection lesion severity (b). The Manhattan plots for the isolates 13-13 and L73A (not shown) also showed no significance, showing only possible, but insignificant, peaks at chromosome 7HS in the 13-13 lesion area, and 3HL in the L73A lesion severity data.

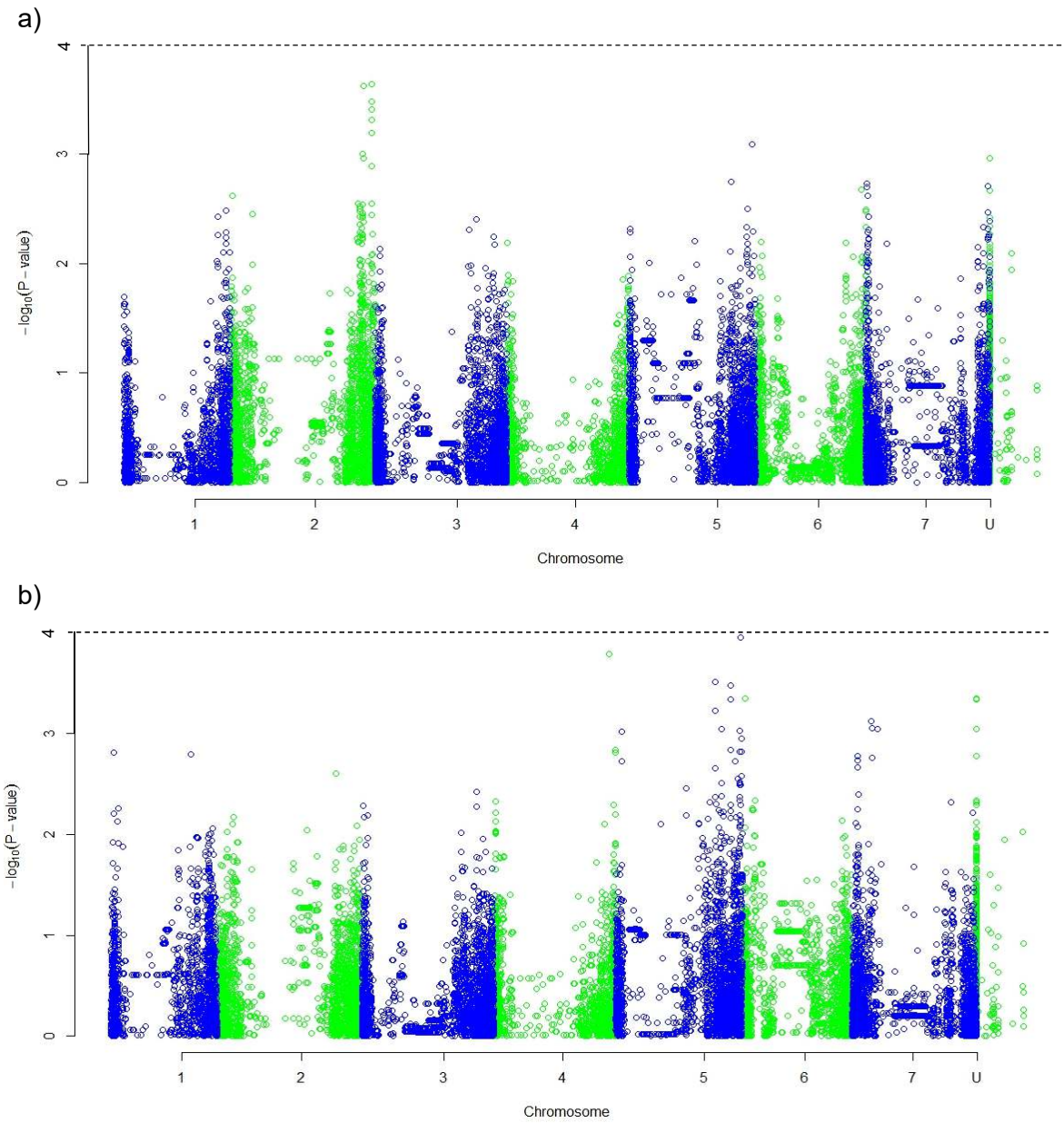


Figure 20) A Manhattan plot of a Genome-Wide Association Study undertaken using a Mixed Linear Model approach on the lesion area **(a)**, and lesion severity **(b)** when infected with *R. commune* (the average data of four isolates); data generated using an ANOVA. Depressions in marker significance observed in the centre of each chromosome are due to reduced marker density around the centromere of the physical map.

The only associations that were above the threshold set were seen with isolate L2A (Figure 21) and L77 (not shown) for both lesion area and lesion severity. Significant associations with isolate L77 are seen and indicated at 5HL in the lesion severity and area data, respectively. Lesion area also shows association in 2HL and 4HL. The strongest associations are in L2A that also show a peak at 5HL for lesion severity, with significant markers (Figure 21b), as well as a peak at 7HS that appears in both lesion area (Figure 21a) and severity data. A further peak is indicated for lesion area in 4HS.

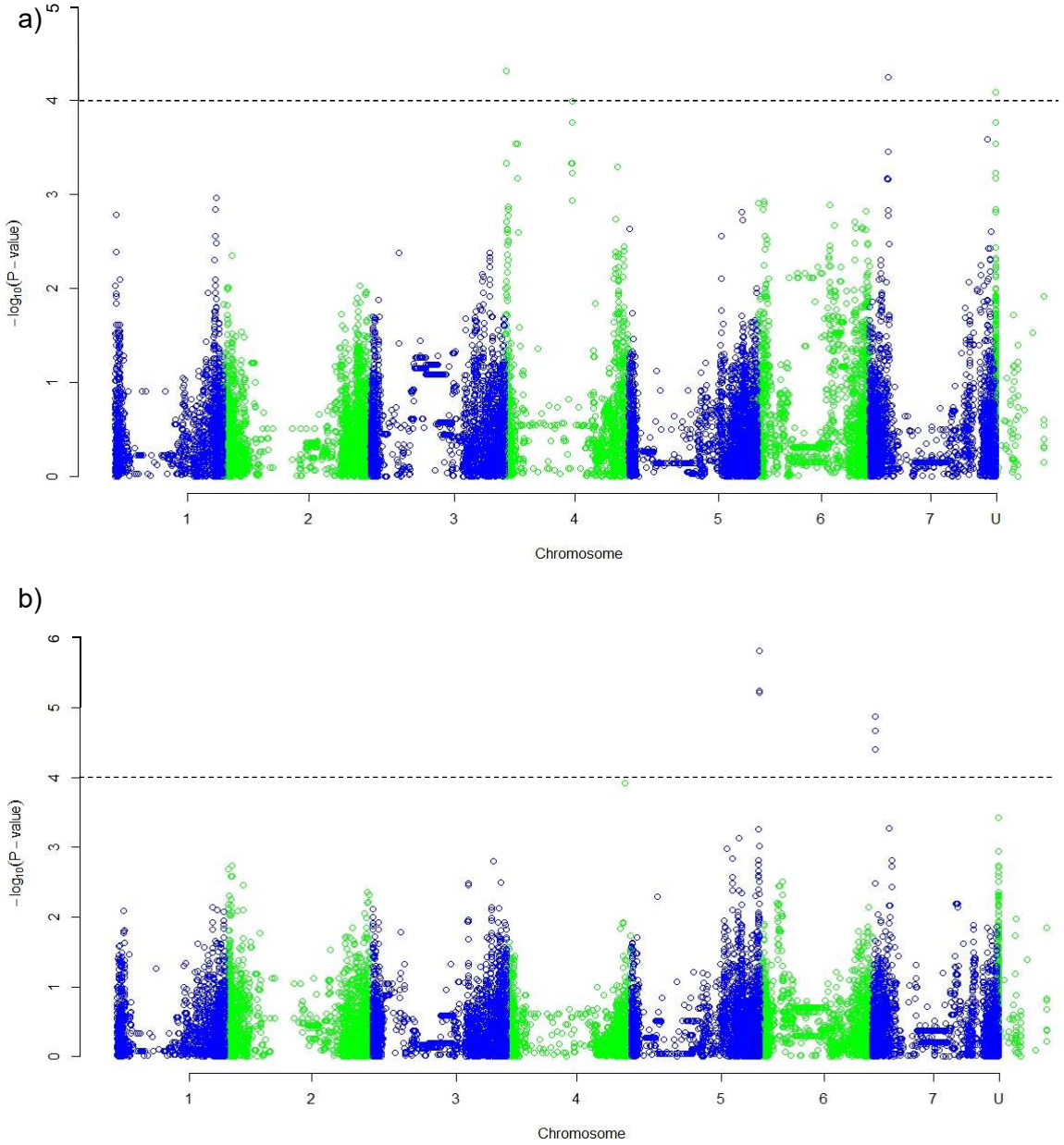


Figure 21) A Manhattan plot of a Genome-Wide Association Study undertaken using an EIGENSTRAT approach on the lesion area **(a)**, and lesion severity **(b)** when infected with *R. commune* isolate L2A; data generated using an ANOVA. Depressions in marker significance observed in the centre of each chromosome are due to reduced marker density around the centromere of the physical map.

Within chromosome 5HL the regions identified do not overlap, the significant markers from isolate L2A lesion severity, L77 lesion area, and L77 lesion severity were positioned between 667.34-667.59 Mb, 621.89-622.53 Mb, and 556.49-570.02 Mb, respectively - the latter along with markers from isolate L73, isolate L2A and the mean lesion severity. Within the region identified with the isolate L77 area data there were a number of genes of interest based on their putative function (Table 8): encoding for a BED zinc finger family protein, and a Bax inhibitor-1 family protein. The most significant region at 667.34-667.59 Mb had only one gene of interest, with the putative function of a receptor kinase with a domain homologous to Ginkbilobin-2 (Gnk2) – an antifungal protein found in *Ginkgo biloba* (Miyakawa *et al.,* 2014). Of these, zinc finger domains are relatively common comprising 1.2% of all annotated genes with known functions in barley, at 902 of the 73586, as listed in BARLEX the barley genome explorer (Colmsee *et al.,* 2015). The Bax inhibitors and Gnk2 domain containing proteins conversely represent <0.1% of genes combined, with 13 and 68 genes found across the genome, respectively.

Table 8) The name of the candidate genes identified in relation to rhynchosporium lesions in detached leaf assays, with the chromosome and position on the physical map listed.

Gene Name	Chr	Position	Annotation
HORVU5Hr1G105590.1	chr5H	621.83	BED zinc finger family protein, expressed
HORVU5Hr1G105760.4	chr5H	622.13	Bax inhibitor-1 family protein
HORVU5Hr1G124810.4	chr5H	667.43	Receptor kinase 1 (Gnk2 domain)
HORVU2Hr1G102740.3	chr2H	697.86	WRKY DNA-binding protein 35

Similarly, chromosome 7HS had two regions: one from the 13-13 area data that had markers, but not significant at 10.15-10.94 Mb, and the other from the L2A lesion severity data with five significant markers at 16.67-16.92 Mb. These could possibly correlate to Rrs2 and Rrs12, respectively. Likewise, the markers identifying a significant region on 4HS in the L2A area data possibly correlate with the location of Rrs16, and the large region on chromosome 4HL between 572.61-596.53 Mb, could co-locate with Rrs3.

The significant marker on 2HL in the isolate L77 area data at 697.86 Mb, possibly correlates to a QTL found by Backes *et al.* (1995) that associated with rhynchosporium and that greatly affected barley kernel yield. The maker is located in a WRKY DNA-binding protein (Table 8) which comprises 77 genes genome wide representing 0.1% of the genome.

Field Trials

The 2016 field trial used a selection of 50 lines of the Bere and other landrace lines available and showed a significant difference in RAUDPC between lines (p-value=0.038). The RAUDPC of the Bere sub-category was approximately 60% of the landrace subcategory. No significant difference between individual lines was identified using the transformed data (p=0.103). However, the untransformed data and the transformed data when the lines were collated both show significant differences (p=0.039 and 0.023, respectively). Individual lines varied greatly, and sub-category ranges overlapped (Figure 22). This allowed for identification of lines that have large levels of infection, such as Bere 23 B and Nepal 92 BN-1, and lines with effectively no infection, such as Bere 8-125 with 0.4% of the RAUDPC of the most infected lines.

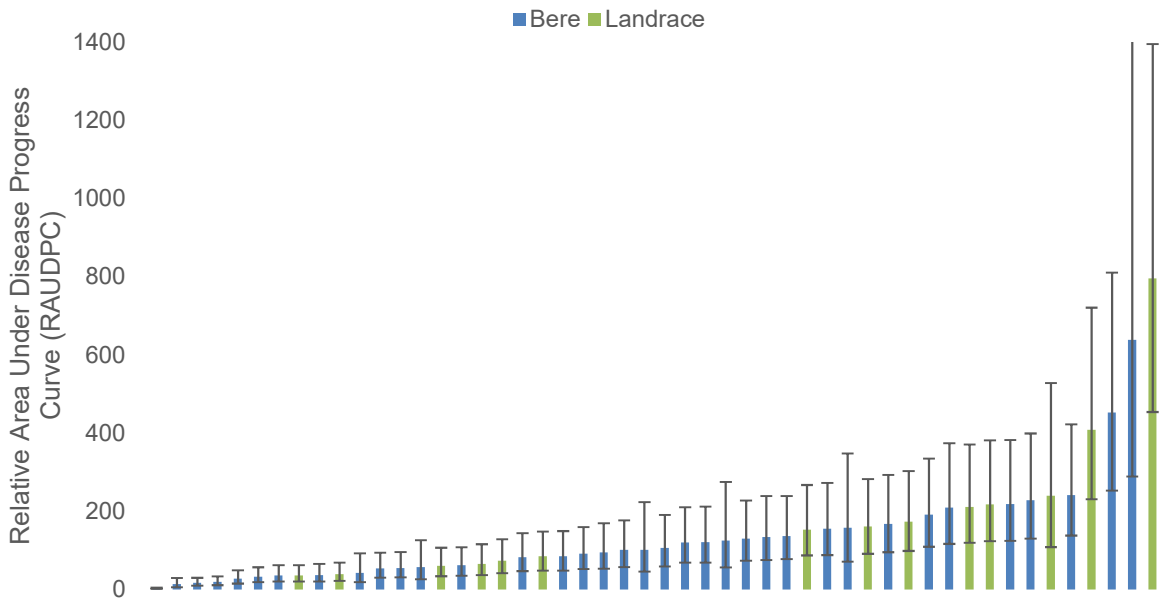


Figure 22) Comparison of rhynchosporium recorded as the Relative Area Under Disease Progress Curve (RAUDPC) during a field trial in 2016 for 50 lines of barley. Error bars represent the standard errors.

Analysis of the transformed 2017 field trial data showed significant differences in the RAUDPC between the sub-categories (p<0.001). The difference between the Beres and other landraces is seen again, with the other landraces showing 50% higher RAUDPC than the Bere lines. However, the elite cultivars (absent in the 2016 field trial) showed an even higher RAUDPC, approximately four times that of the Bere lines (Figure 23).

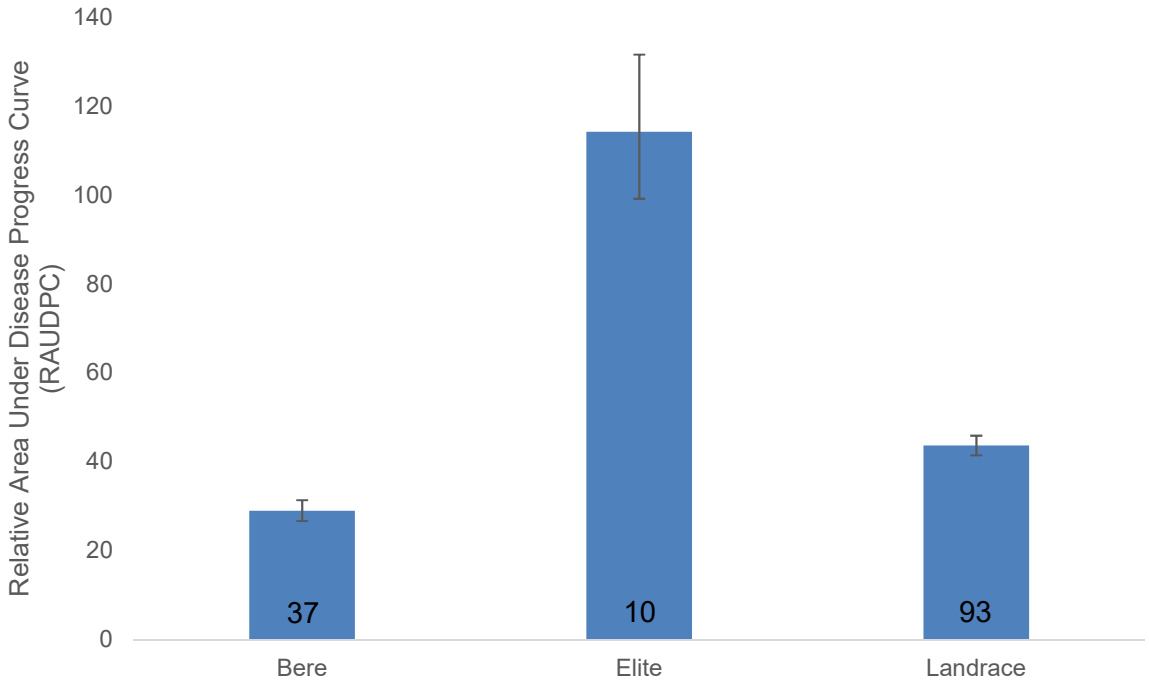


Figure 23) Comparison of rhynchosporium recorded as the Relative Area Under Disease Progress Curve (RAUDPC) during a field trial in 2017 for 140 lines/cultivars of barley divided into three, unequal, sub-divisions (37, 93, and 10 for the Bere, other landraces, and elites respectively). Error bars represent the standard errors.

Analysis of the individual data similarly showed a significant difference between the lines/cultivars ($p<0.001$). The Bere line, Bere 8-125, identified with a low RAUDPC value in the previous field trial similarly performs well in this trial. One Bere line had a smaller value than this line, Bere 25 A, but this had an average value for a Bere line in the 2016 trial. This difference between field trial years is also found with other Bere lines such as Bere 58 A 36 Eday, Bere 53 A 31, Bere-223, and Bere 37 A 14. A larger spread of the other landraces can be seen, in part because there is a greater number with both high and low RAUDPC values. The three landraces with the smallest RAUDPC value were Danubia, Isaria, and Burtons Malting-128, comparable to that of Bere 8-125 but none of these lines were in the 2016 trial to compare. As identified in the sub-category analysis the elite cultivars are amongst those with the highest RAUDPC value, with all elite cultivars above the median (Figure 24).

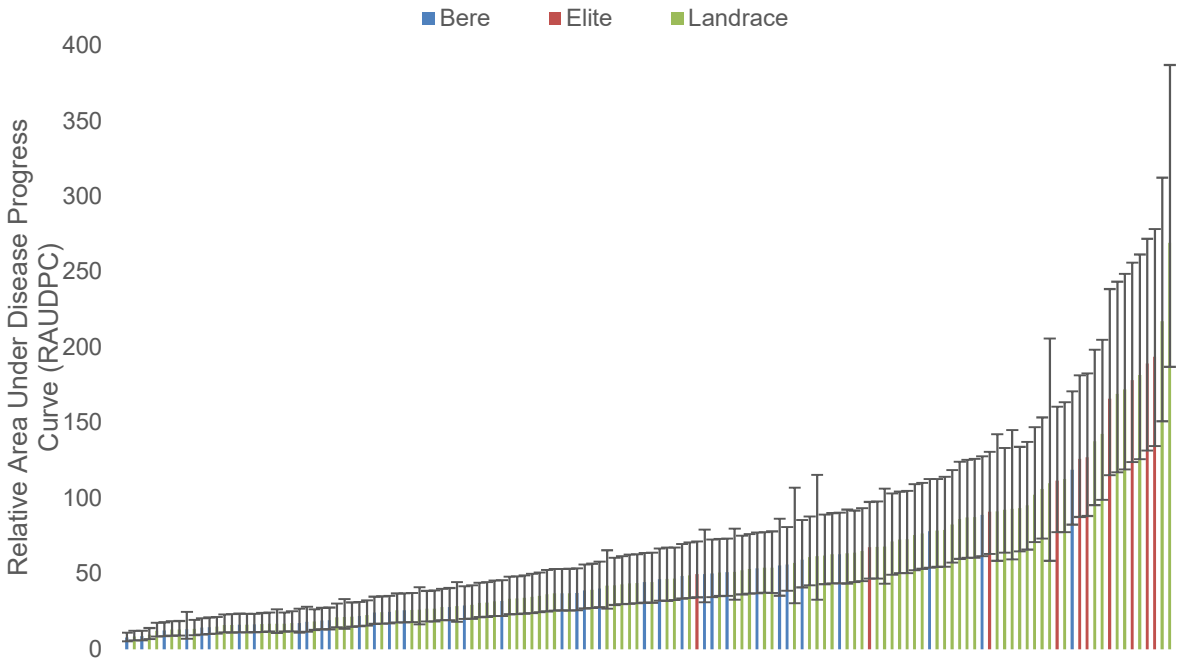


Figure 24) Comparison of rhynchosporium recorded as the Relative Area Under Disease Progress Curve (RAUDPC) during a field trial in 2017 for 140 lines/cultivars of barley. Error bars represent the standard errors.

A comparison of the lines over the two field trials (Figure 25) shows no correlation between the two years (correlation coefficient>0.26). However, this allowed for identification of lines that had low RAUDPC in both years. Of these Bere 8-125 stood out clearly, other included Bere (Mr SO)-121 and Bere-155.

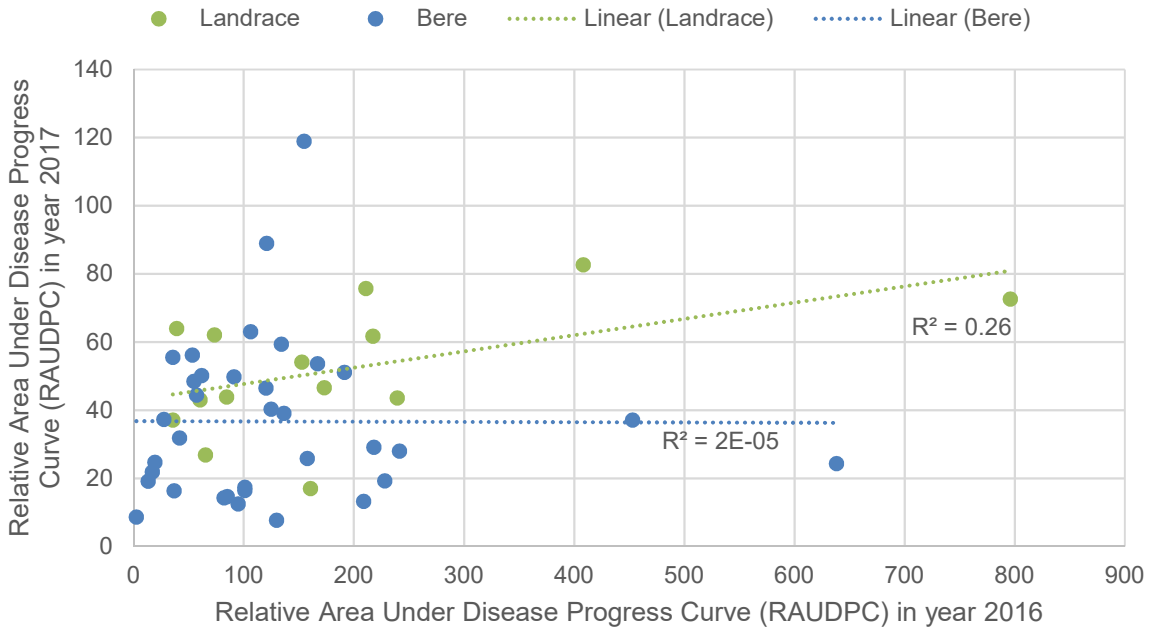


Figure 25) Correlation of rhynchosporium recorded as the Relative Area Under Disease Progress Curve (RAUDPC) between field trials in 2016 and 2017, for the 49 line in common split into two sub-categories of Bere and other landraces (with 35 and 14 line respectively). For each sub-category the line of best fit along with the coefficient of determination (R^2) value is given; P-values= 0.063 (Landrace) and 0.981 (Bere).

1.1.1.13. Comparison with Controlled Study

Comparison of the field trial data from both years with the detached leaf assay average lesion area and lesion severity (Figure 26) shows no significant correlations between the datasets. However, from these correlations three Bere lines were identified as having consistently low levels of infection: Bere 45 A 23, Bere 58 A 36 Eday, and Bere 8-125.

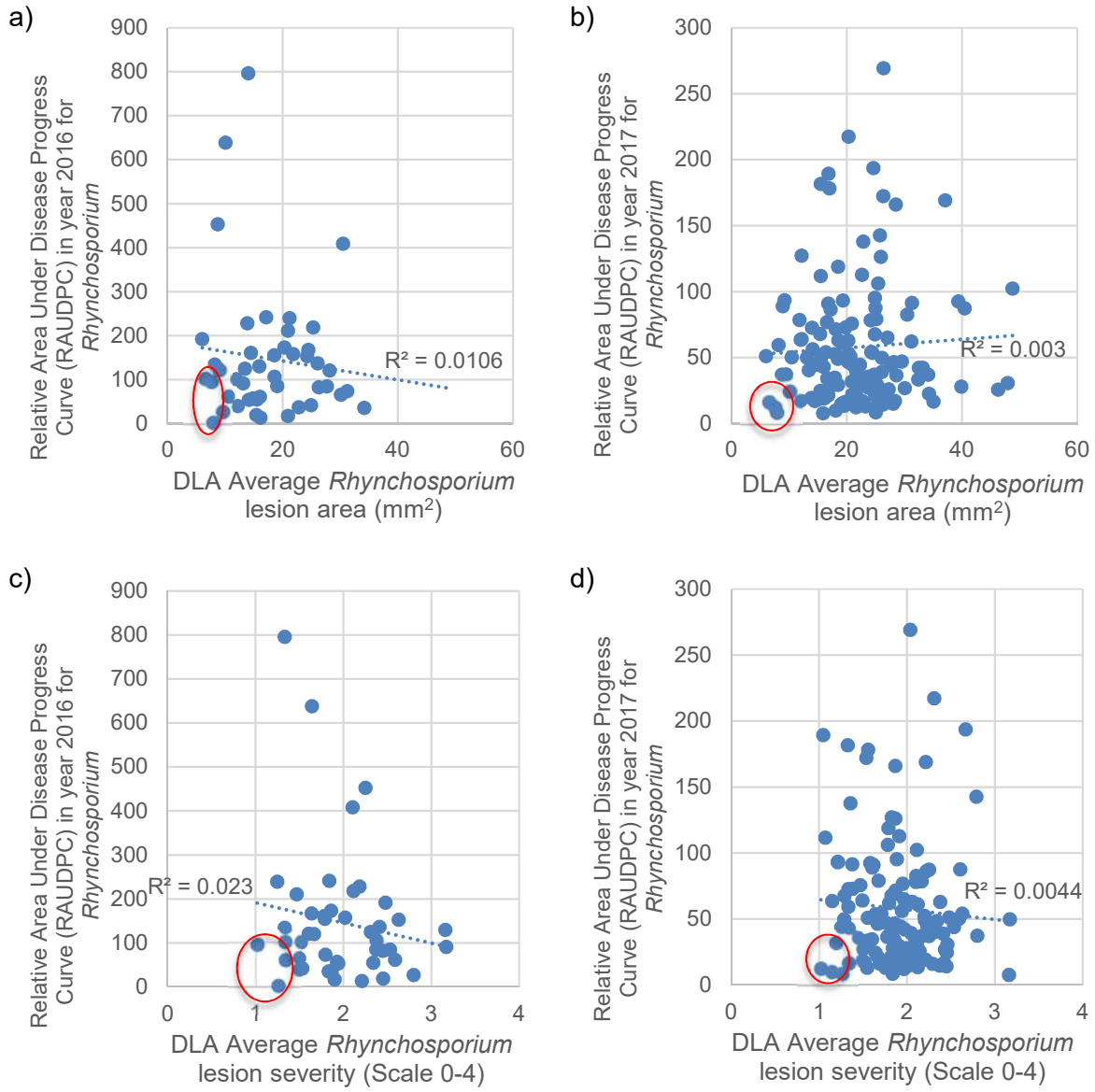


Figure 26) Correlation between the DLA average lesion area (**a** & **b**) or lesion severity (**c** & **d**) with the field trial RAUDPC from the years 2016 (**a** & **c**) or 2017 (**b** & **d**) with the respective 50 or 140 lines/cultivars used. The three lines – Bere 45 A 23, Bere 58 A 36 Eday, and Bere 8-125 – that were shown to have consistently low infection are circled in red. For each correlation the line of best fit along with the coefficient of determination (R^2) value is given. P-values= 0.497, 0.534, 0.314, and 0.45, respectively.

1.1.1.14. Genome-Wide Association Study (GWAS) Analysis

Of the 37242 markers used with the 2017 field trial data, 10718 were removed as having low minor allele frequency and a further 42 because of a low call rate. Of the 130 lines used 13 were excluded because their heterozygosity was too high, and a further eight due to being identical by state. The QQ plot showed the Mixed Linear Model approach had the lowest deviation from the expected null distribution. The Manhattan showed one peak that stands out significantly on the distal end of chromosome 3HS in the region of 44.46-45.33 Mb (Figure 27).

Within this region there were no genes with a putative function that, to date, is known to affect pathogen infection. However, next to this region – at 44.42 Mb, less than 0.5 cM – is a Cysteine-rich receptor-like protein kinase with a domain homologous to Gnk2 mentioned above (HORVU3Hr1G017430.1). One significant marker, with the highest effect, is found on chromosome 2HL at 713.11 Mb (not in the same area as found the DLA data) within a gene encoding for a disease resistance protein with leucine rich repeats (HORVU2Hr1G108000.2).

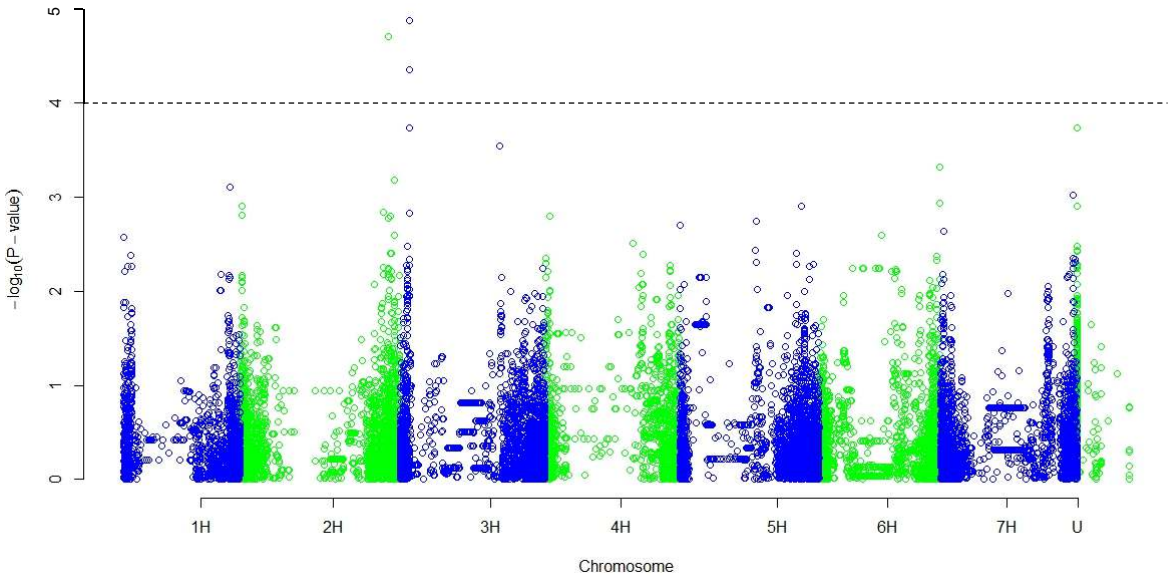


Figure 27) A Manhattan plot of a Genome-Wide Association Study undertaken using a Mixed Linear Model approach on Relative Area Under Disease Progress Curve (RAUDPC) of rhynchosporium during a 2017 field trial; data generated using an ANOVA. Depressions in marker significance observed in the centre of each chromosome are due to reduced marker density around the centromere of the physical map.

Discussion

Barley scald caused by the fungal pathogen *Rhynchosporium commune* is a global disease, and a major problem in most parts of the UK (Avrova and Knogge, 2012). It is important to find new sources of resistance as resistance genes cause a selective pressure on the pathogen to develop a mechanism to break the resistance of the plant (Bergelson *et al.,* 2001). Resistance genes that have developed in secluded populations, such as landraces, pose a potential source of novel resistance genes that can be bred into the elite cultivars (Silvar *et al.,* 2010). This project assessed landrace lines for rhynchosporium symptoms in both detached leaf assays and field trials. This allowed for identification of a number of Bere lines that showed signs of resistance, as well as identifying regions within the genome that correlated to reductions in infection symptoms, along with a number of genes with putative functions associated with disease resistance.

Controlled Study Symptoms

The detached leaf assays performed on the JHI-SBLC showed that there is a range of infection for all sub-categories, with overall the Bere lines showing smaller lesions, but with more necrotic tissue in the lesion. These are comparable to the study from Coulter *et al.* (2018) that shows identified resistant lines with lesions reduced by 45% compared to the most susceptible line Morex. This study also identified Morex as one of the most susceptible lines, with the largest lesions, and identified many other lines with similar levels of lesion size reduction as found in the Coulter *et al.* (2018) study when compared to Morex. The smallest lesions in this study were reduced by 78% compared to Morex, with the three lines identified for consistent resistance – Bere 45 A 23, Bere 58 A 36 Eday, and Bere 8-125 – reduced by 71-76% compared to Morex. This supports the anecdotal claims of farmers working with Bere populations that they were more resistant to rhynchosporium (Mahon *et al.,* 2016). However, these results show that there is a large difference between how different isolates interact with individual lines, this is highlighted by the isolate 13-13 that interacts with lines/cultivars in a differential manner from the remainder of the isolates. Difference seen in the lesion severity are possibly due to differences in reaction/susceptibility to necrosis inducing peptides from the pathogen, as *R. commune* has not been shown to induce a hypersensitive response (Wevelsiep *et al.,* 1991; Hahn *et al.,* 1993).

Currently there are multiple rhynchosporium resistance genes that are spread across all barley chromosomes, except 5H (Hanemann, 2009). The identification of new resistance genes that can be introgressed into elite lines will help make a more robust resistance. This study identified several regions across multiple chromosomes that are associated with one or more of the isolates with regards to lesion area and/or lesion severity. In the DLA this included regions that are near the location of previously identified Rrs genes, including: two peaks on chromosome 7HS from the isolate 13-13 lesion area data and the L2A lesion severity data that could correlate with Rrs2 and Rrs12, respectively (Abbott *et al.,* 1992; Hanemann*,* 2009), one peak on 4HS from the isolate L2A lesion area data possibly correlating with Rrs16 (Pickering *et al.,* 2006), and one peak on chromosome 4HL also from the isolate L2A lesion area data close to the estimated region of Rrs3 (Bjørnstad *et al.,* 2002). The two regions of interest identified on chromosome 2HL and 5HL, the latter of which has not had a Rrs gene identified on that chromosome before and the former has not had one identified in that region (Zhan *et al.,* 2008).

Within the region on chromosome 5HL there were three proteins with putative functions that were identified as potentially responsible for the reduction in symptoms included: a) BED zinc finger family protein – selected as this type of zinc finger has been associated with R genes in barley (Gupta *et al.,* 2012), b) Bax inhibitor-1 (BI-1) family protein – selected as it has been shown that a wheat BI-1 gene, that was shown to be differentially expressed when the wheat was inoculated with *Fusarium graminearum*, and caused increase resistance to a pathogen when expressed in *Arabidopsis* (Lu *et al.,* 2018), and c) a receptor kinase with a domain homologous to Ginkbilobin-2 (Gnk2) – selected as Gnk2 is an antifungal protein found in *Ginkgo biloba* causing the inhibition of the growth of fungal pathogens such as *Fusarium oxysporum* (Miyakawa *et al.,* 2014).

The marker identified on chromosome 2HL was located within a WRKY DNA-binding protein that have been shown to be involved in the defence against both biotic and abiotic stresses (Agarwal *et al.,* 2011; Zhu *et al.,* 2013) such as the suggested response to powdery mildew in barley (Wang *et al.,* 2014a).

Field Trial Symptoms

The field trial results corroborated that Bere lines showed diminished symptoms. The spread of the lines/cultivars shows that there were large amounts of variation within each sub-category with regards to resistance to rhynchosporium, with individual Bere and other landrace lines being identified in both years as having both low and high levels of disease. Whilst visual scores are not directly numerically comparable to scores done by others, they can still be generally compared. A similar experiment, from Looseley *et al.* (2018), undertook trials in the same fields over three years with common elite cultivars to assess European spring barley germplasm. As in this study, Propino consistently showed amongst the lowest infection of the common elite cultivars, though in contrast so did Belgravia. The most resistant lines from each year of the Looseley *et al.* (2018) showed up to a 54% reduction in RAUDPC compared to Propino. In contrast, the most resistant line in the 2017 trial showed a reduction of 84% compared to Propino, with the three lines identified for consistent resistance mentioned above showing 67-83% reduction compared to Propino. This variation could be due in part to the different environmental conditions found between the origins of the lines, with some coming from heterogenous populations (Ceccarelli *et al.,* 1987; Yitbarek *et al.,* 1998). However, the elite cultivars showed much higher levels of infection than the DLA suggests. This could be explained by the difference in height, as the effect of the dwarfing genes in elite lines give smaller distances between the leaf nodes making it more likely for the spores to spread by splash up onto higher leaves (von Korff *et al.,* 2005; Looseley *et al.,* 2012). Additionally, there was no correlation between the same lines when grown in 2016 and 2017 when comparing infection levels, possibly due to different environmental conditions and/or different *R. commune* isolates in the field (Looseley *et al.,* 2015; Looseley *et al.,* 2018). This could highlight the need for increased discovery of resistance mechanisms to provide a broad protection to the unpredicted annual changes in infection and infecting isolates in the same field.

Using the data from the 2017 trial, one genomic region was found to be significantly associated with the difference in symptoms on chromosome 3HS, along with a significant marker on chromosome 2HL. Neither region has any known Rrs gene, and thus may be evidence of potential sources of novel resistance genes. The region found in 3HS was next to a Cysteine-rich receptor-like protein kinase

with a domain homologous to Gnk2, the marker at 2HL was located in a disease resistance protein with LRRs – these were selected for the reasons mentioned above (Hammond-Kosack and Jones, 1997; Miyakawa *et al.,* 2014). This region was less than 0.5 cM away from the significant markers, within the minimum distance of 4 cM in which linkage disequilibrium has a low rate of decay in elite barley (Bengtsson *et al.,* 2017; Zhou *et al.,* 2012b).

No correlation was found between the DLA and field data, again possibly due to architectural differences of the whole plant influencing the number of spores able to splash up the plant (von Korff *et al.,* 2005; Looseley *et al.,* 2012), or due to differences in environmental conditions. Other possible explanations could be that the isolates found in the nursery were dissimilar to those used in the DLA experiment. However, three Bere lines were identified as having consistently low levels of infection: Bere 45 A 23, Bere 58 A 36 Eday, and Bere 8-125. As these lines had low infection in the DLA it shows that it is more than a plant architecture difference that is causing the low infection scores in the field for these lines. Thus, these lines show potential for novel sources of resistance.

Future Work and Implications

Proposed future work would be to test these lines in a fully replicated large plot field trial along with DLAs with additional isolates, to further assess the level of resistance of these lines. Further analysis is also necessary to identify whether the resistance exhibited in these lines is, in part or in whole, caused by the identified chromosomal regions of interest in 2HL, 3HS and/or 5HL, with emphasis on the candidate genes identified. This could be achieved by creating a bi-parental mapping population to fine map the regions of interest. If these regions are the cause of this increased resistance, they could continue to be introgressed into elite breeding lines to develop new cultivars. This would help protect the elite crops from rhynchosporium infection by providing an additional form of resistance that the pathogen would need to overcome. The development of cultivars with a robust resistance to all isolates of rhynchosporium is important especially with the potential of ongoing human caused climate change resulting in differing selective pressures on both the crop and the pathogen, that may promote the evolution of *R. commune* to break resistance or that may weaken the crop making it more susceptible (Stefansson *et al.,* 2013; Velásquez *et al.,* 2018).

Assessment of the interaction of dual biotic and abiotic stresses in the Scottish barley landrace Bere (*Hordeum vulgare* L.)

Introduction

Abiotic and biotic stresses do not occur independently as plants will experience near constant exposure to both types of stress in both natural environments and agricultural environments. Therefore, common pathways for multiple stresses have developed. Prime examples of this are the hormone signalling pathways such as abscisic acid (Mauch-Mani and Mauch, 2005; Asselbergh *et al.,* 2008) and jasmonic acid, along with the associated pathways of ethylene and salicylic acid (Lorenzo and Solano, 2005) that all have an associated role in response to and limitation of both biotic and abiotic stress, allowing them to respond to multiple stresses concurrently (Bostock, 2005). This is thought to be due to the fact that responses to stress often overlap, encoding for similar sets of transcription factors, and thus there is the need for cross-talk between the different signalling pathways (Figure 28) (Fujita *et al.,* 2006).

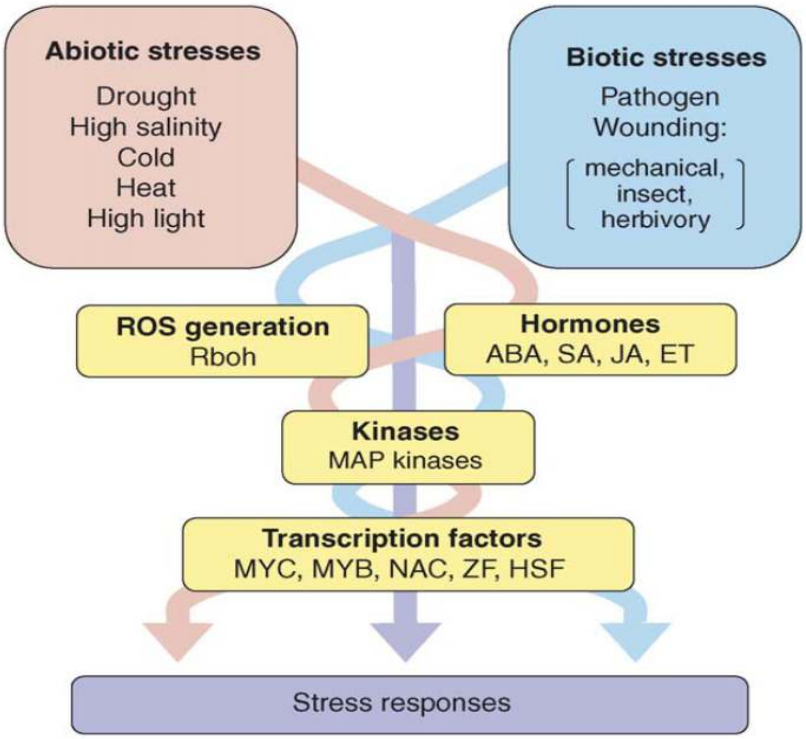


Figure 28) A representation of the convergence points in abiotic and biotic stress signalling pathways that formulate a common stress response. Sourced from Fujita *et al.* (2006).

Stress responses, however, are often antagonistic to one another, with different pathways being involved in both increases in susceptibility or resistance (Anderson *et al.,* 2004). This is well represented in the trade-off between biotrophic and necrotrophic fungi, with pathways involved in increased resistance to one causing an increased susceptibility to the other. This can be caused by the hypersensitive response (HR) manifest through the salicylic acid pathway causing the reduction of biotrophic pathogens, but providing resources for necrotrophic pathogens, whereas the antagonistic jasmonic acid pathway causes the opposite to happen (Kliebenstein and Rowe, 2008; Robert-Seilaniantz *et al.,* 2011). Similarly, the *Mlo* gene has been shown to negatively regulate plant defences, and the loss of function mutation has provided a widely used resistance to powdery mildew. This resistance, however, comes at a cost of increasing susceptibility to necrotrophs such as *Ramularia collo-cygni*, the causal agent of ramularia leaf spot (McGrann *et al.,* 2014). Another example of antagonist effects in combined stresses is seen in the semi-dwarfing 'uzu' barley, the mutation causes a disruption to the Brassinosteroid hormone. This disruption results in an increased resistance to a range of pathogens, but also results in an intolerance to certain abiotic stresses such as cold and drought (Ali *et al.,* 2014; Goddard *et al.,* 2014). There is also evidence that the plant response to stress combinations is different from the combination of the individual plant stresses, resulting in different transcriptome patterns being produced (Rizhsky *et al.,* 2004).

Interaction of stresses also occurs when the effect of one stress causes an impact on the effect of another stress. This is commonly seen in plants that are abiotically stressed having weakened defence mechanisms becoming more susceptible to pathogen infection (Suzuki *et al.,* 2014). The study of abiotic stress effects on biotic stress has been shown to be both positive and negative (Atkinson and Urwin, 2012). Commonly, temperature stress has a negative effect by increasing susceptibility to pathogens, this is seen in the example from Sharma *et al.* (2007) that showed an increase in the fungal disease spot blotch in wheat with elevated night time temperatures. Other abiotic stresses have been shown to increase the resistance of a plant to particular pathogens. The study from Wiese *et al.* (2004) showed that increasing levels of salt correlated with increasing resistance to the powdery mildew disease in barley even at low salt stress levels and remained for

some time after the stress was relieved. The study showed that the salt stress induced a papilla-mediated resistance that blocked the fungus, similar to that mediated by the *mlo* gene. This will change in less predictable ways as a result of climate change. Differences in the climate change associated stresses carbon dioxide levels, ozone levels, and temperature will cause changes in the metabolome of the plants resulting in differences in pathogen infection. These stresses will occur in conjunction with each other and may result in altered reactions from those found when assessing the stresses individually. Mikkelsen *et al.* (2015a) showed increased resistance to powdery mildew when grown with increased stress of the individual climate change associated stresses, but not when these stresses where applied in conjunction. Similarly, the reverse is shown for spot blotch disease (Newton *et al.,* 2011b; Newton *et al.,* 2012; Mikkelsen *et al.,* 2015b).

Metals play a role in the response to biotic stresses in plants, being both essential for attack and defence mechanism. Thus, it is apparent that metal stresses influence how biotic stress is manifest within the plant system. However, it becomes more complex as metal ion concentration affect both the plant susceptibility and the pathogen virulence (Poschenrieder *et al.,* 2006). This is also seen in reverse, with the pathogen having an effect on the plant system, triggering either increases or decreases in metal absorption (Tamás *et al.,* 1997). An example of the former is seen in the difference in interaction of manganese and plant pathogens in a number of different species. The review by Huber and Wilhelm (1988) showed increase Mn in barley causes increases in leaf spot disease, decreases in aphid infection, and both in different mildew infection studies. The metalloid silicon (Si) has shown to have particular importance in stress resistance, causing increased resistance to both biotic and abiotic stresses. This has been demonstrated by the increased susceptibility to other stresses when deficient, such as the increase in powdery mildew in Si deficient barley and wheat, and reversely the increase in resistance when supplemental Si was applied (Ma*,* 2004).

Clearly it is important to study stresses together and how they interact in the environment. This is critical in the development of stress resistant crops, as there may be hidden costs associated with the resistance when in conjunction with other stresses. Additionally, breeding for resistance to one stress could result in resistance to a further stress. Together these provide both concern and opportunity to

breeders to breed robust crops that are able to maintain yield under adverse environmental conditions. This is especially important with respect to climate change as the associated fluctuations in abiotic conditions – such as temperature, light, water, carbon dioxide and nutrient availability – will not only cause a change in the levels of biotic stresses, but will also change how these stresses interact potentially causing an unpredictable level of damage (Atkinson and Urwin, 2012). As stated above, these changes will be more unpredictable due to the complex interaction of the abiotic stresses producing differing metabolomes with different resistances and susceptibilities to the different pathogens (Newton *et al.,* 2012; Mikkelsen *et al.,* 2015b).

The aim of this study was to assess the interaction of the stresses analysed in previous sections, using one abiotic stress (salinity or manganese deficiency) to stress the plant tissue and to then apply a biotic stress (rhynchosporium) and observe how these symptoms differ from the unstressed controls. This was undertaken with a number of different lines showing contrasting levels of resilience to one or more of these stresses. It was suspected that there would be differences in rhynchosporium symptoms between the different applications of the two abiotic stresses as both manganese (Huber and Wilhelm, 1988) and salt stress (Wiese *et al.,* 2004) have been shown to affect fungal infections. It was unclear as to whether these abiotic stresses would increase or decrease symptoms as the differences between the pathogens tested and *R. commune* could cause differences in reaction, such as the difference between biotrophic and necrotrophic pathogens.

Results

Mn-Rhynchosporium Interaction

The analysis of the lesion area, after transformation, shows a significant difference between the soil types ($p=0.007$), lines/cultivars ($p<0.001$), and interaction of the treatments ($p<0.001$). This data (Figure 29) revealed that almost all the Bere lines that had significant differences in lesion size between the two soils had larger lesions in the Bullion soil, with the exception of Bere 59 A 3 Uist that had larger lesions in the soil from Orkney. A similar trend was found in the elite cultivars, with Scholar and Propino having the largest lesions in the Bullion soil but some of the smallest in the soil from

Orkney, and the opposite for the cultivar Westminster. The only other landrace lines that showed differences were Prior-195 and Scottish Annat-202 that both, along with Bere 59 A 37 Uist, had the three largest lesion size in the Orkney soil but small lesion sizes when grown in Bullion soil. However, there was no correlation with the lesion size, in plants grown in either of the soils, and the lesion size from 13-13 inoculation in the single interaction DLA study in the Mn landrace screen. There is also no correlation with the lesion sizes from either soil or either of the chlorophyll fluorescence measurements.

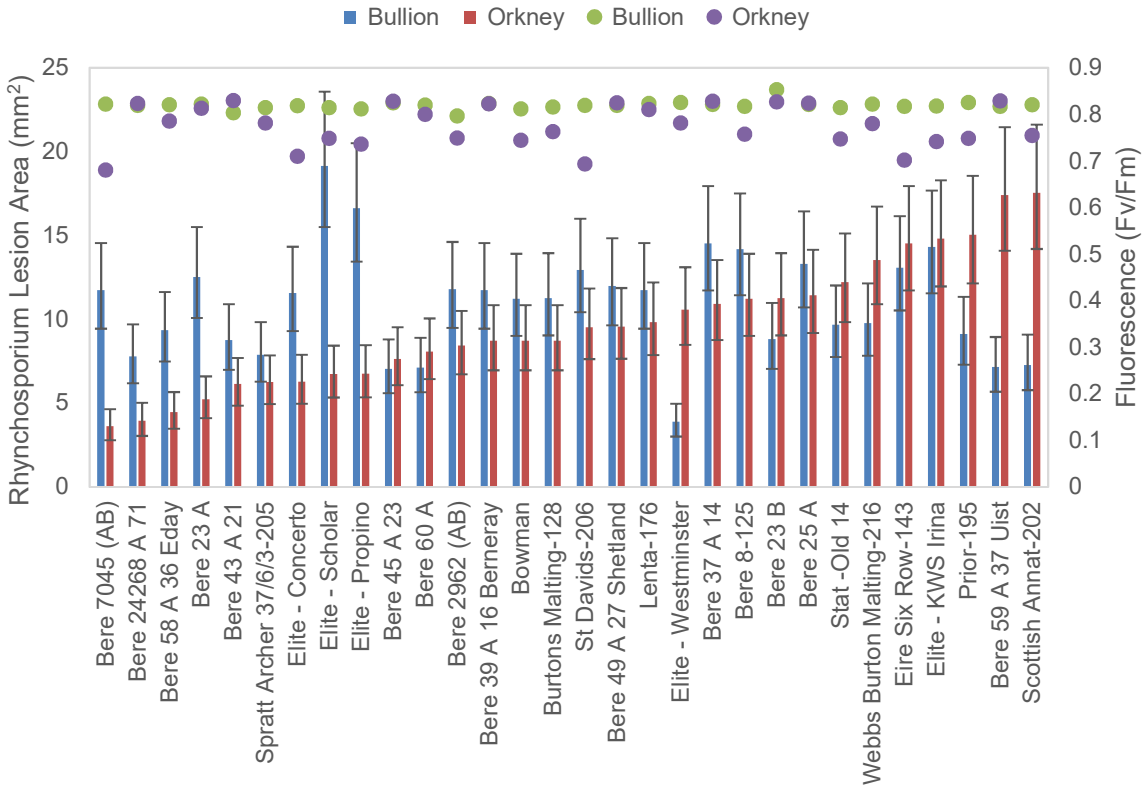


Figure 29) Comparison of the rhynchosporium lesion area for 30 lines/cultivars of barley, using isolate 13-13, grown in two different soil type: Orkney soil that is Mn limited, and Bullion soil that has adequate Mn (displayed as red and blue bars respectively on the primary axis). The secondary axis displays the mean chlorophyll fluorescence of the plants grown the two soil types (displayed as purple and green points). Error bars represent the standard errors in positive and negative directions.

The analysis of the lesion severity did not show a significant difference between the soil types ($p=0.542$), but did between the: lines/cultivars ($p=0.001$), and their interaction ($p<0.001$). Comparison of the lesion severity data (Figure 30) shows fewer differences between the two soil types, with only the Bere lines Bere 59 A 37 Uist, Bere 60 A and Bere 7045 (AB) showing differences, the former of which was also the Bere with the largest lesions in Orkney soil. Bere 60 A and Bere 59 A 37 Uist

showed more severe lesions when grown in Orkney soil, whilst Bere 7045 (AB) showed the opposite. Again, a similar pattern is seen with the elite cultivars, Scholar and Concerto having more severe lesions in Bullion soil and KWS Irina showing the opposite. The landrace Prior-195, but not Scottish Annat-202, showed increased lesion severity when in Orkney soil, as seen in the lesion area data.

As with the lesion area, there was no correlation with the lesion severity in plants grown in either of the soils, and the lesion severity from 13-13 inoculation in the single interaction study in the Mn landrace screen. However, there was a weak positive correlation (R^2 = 0.42) between the lesion severity and chlorophyll fluorescence of plants grown in soil from Orkney. This is not seen in the other comparisons of lesion severity and chlorophyll fluorescence.

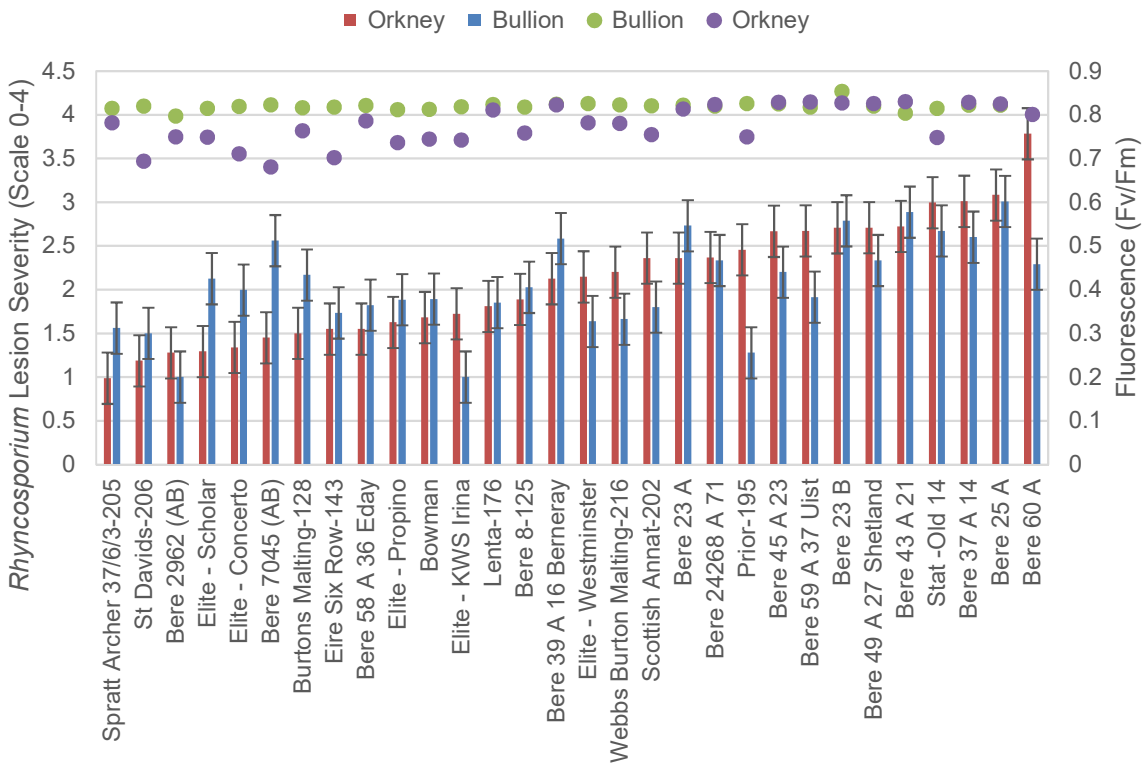


Figure 30) Comparison of the rhynchosporium lesion severity for 30 lines/cultivars of barley, using isolate 13-13, grown in two different soil type: Orkney soil that is Mn limited, and Bullion soil that has adequate Mn (displayed as red and blue bars respectively on the primary axis). The secondary axis displays the mean chlorophyll fluorescence of the plants grown the two soil types (displayed as purple and green points). Error bars represent the standard errors in positive and negative directions.

Salt-Rhynchosporium Interaction

The analysis of the lesion area, after transformation, show significant differences between the salt concentrations, line/cultivar, and interaction of the treatments ($p<0.001$). Comparisons of the averages show lesion sizes of 11.6 and 16.1 mm^2 for the plants grown on control and the salt treated compost, respectively – an increase of over a third when grown in salt treatments. Analysis of individual lines (Figure 31) reveal that for those that show differences in lesion size between the salt concentration most have an increased lesion size when grown in compost with salt, this includes the two elite cultivars, Bere-118, and other landraces such as Scottish Common-M08 that had nearly triple the size of an already comparatively large lesion size when grown in salted compost. The only two lines to show a significant decrease in lesion size when grown in salted compost were the lines BW 902 and Aramir-M08, that both had amongst the smallest lesions in saline conditions, but the largest lesion sizes when grown in the control compost. However, there was no correlations between the lesion size from 13-13 inoculation in the single interaction study in the Mn landrace screen and the lesion size for each of the salt concentrations, nor a correlation between the lesion sizes of the two concentrations or between the change in dry weight with increasing salt concentrations in the single interaction study in salt landrace screen and the lesion size for each of the salt concentrations.

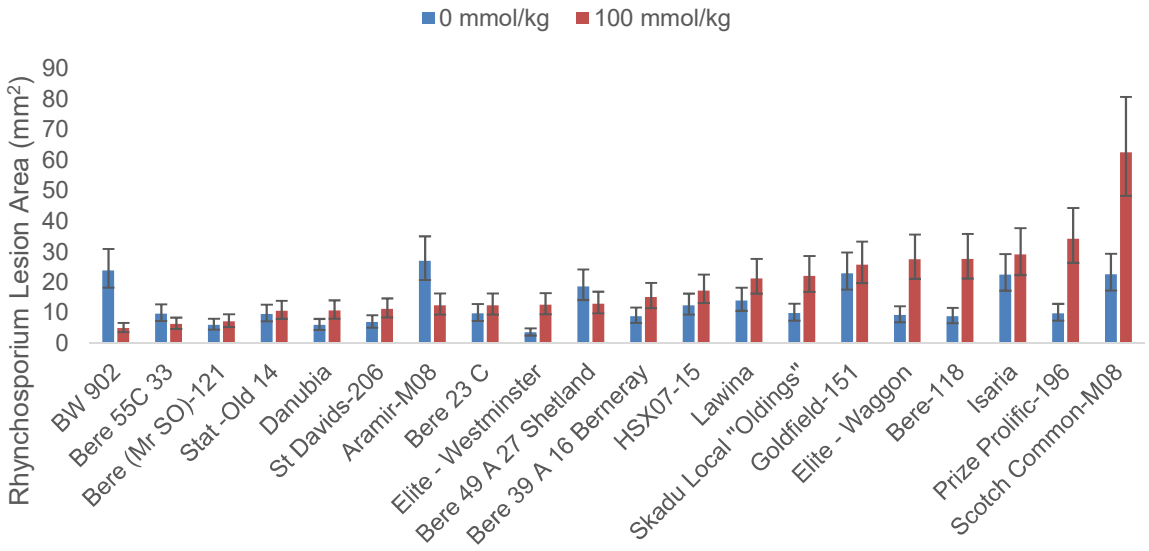


Figure 31) Comparison of the rhynchosporium lesion area for 20 lines/cultivars of barley, using isolate 13-13, grown in universal compost with two different salt concentrations. Error bars represent the standard errors in positive and negative directions.

Analysis of the lesion severity did not identify a significant difference between the salt concentration ($p=0.770$), but did between the lines/cultivars ($p<0.001$), and their interaction ($p<0.001$). The lines with more severe lesions in the compost control are all non-Bere landrace lines – Aramir-M08, Goldfield-152, and Danubia – all of which had the least severe lesions in the salted compost, but average lesion severity scores in the control (Figure 32). The lines with increased lesion severity in the salted compost compared with the control, were Bere (Mr SO)-121, Skadu Local "Oldings", and Scottish Common-M08. The latter had the most severe lesions in the salted compost and a large increase from the control, similar to the lesion area.

Like the lesion area data, no correlations were found in lesion severity between the salt concentrations, nor between these and: the lesion severity from 13-13 inoculation in the single interaction study in the Mn landrace screen, or the change in dry weight with increasing salt concentrations in the single interaction study in the salt landrace screen.

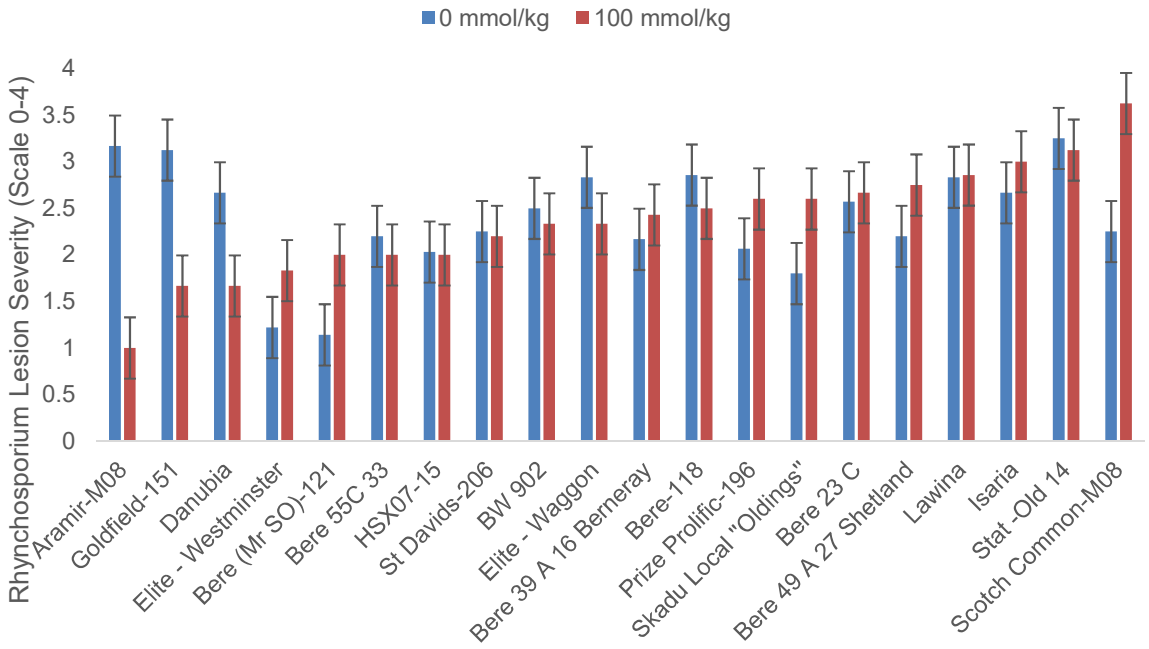


Figure 32) Comparison of the rhynchosporium lesion severity for 20 lines/cultivars of barley, using isolate 13-13, grown in universal compost with two different salt concentrations. Error bars represent the standard errors in positive and negative directions.

Discussion

The effects of abiotic stresses have been shown to both increase and decrease the susceptibility of plants to a range of different pathogens. This is also seen when prior abiotic stress has occurred, resulting in a predisposition due to weakened defence systems, or an enhanced tolerance to stress due to transcriptomics, biochemical, or epigenetic changes (Pandey *et al.,* 2017). This study has demonstrated the complexities of the interaction between one abiotic stress (salinity or Mn deficiency) and one biotic stress (rhynchosporium). The abiotic stresses both caused line dependant changes compared with the control environment, showing clear differences in rhynchosporium symptoms when the stress was applied. Salt stress showed a clear average increase in symptoms when grown in salt, with most lines demonstrating this but some breaking the trend.

Mn-Rhynchosporium Interaction

The inoculation of *R. commune* on detached leaves from selected lines that had been grown in either Mn sufficient or deficient soils showed individual lines/cultivars could be identified as having increased and decreased resistance to rhynchosporium in response to changes in Mn. However, this could not be linked with Mn stress as there was no correlation with the chlorophyll fluorescence data, an indicator of Mn stress. Previously, Huber and Wilhelm (1988) highlighted differences in Mn causing both increases and decreases, both between and within different diseases in barley and other crops. The lack of correlation with the DLA of the single interaction rhynchosporium study, using isolate 13-13, suggests that these data may also be too variable to get meaningful comparisons from. However, this could be a reflection of the large difference in disease expression on leaves grown in hydroponics and leaves grown in soil. Similar results were seen in the lesion severity data, with the key exception of a weak correlation between the lesion severity and the chlorophyll fluorescence when grown in the Mn deficient soil from Orkney. This correlation could suggest that the lesion severity is positively correlated with Mn-tolerance, showing increased tolerance to Mn resulting in an increase in lesion severity. This is the opposite of what was been indicated as the overall trend in Huber and Wilhelm (1988) that suggests that tissues with reduced Mn are more susceptible, but this was a generalisation with many exceptions. Further, these differences could be due to other differences between the two

soils that impact plant performance and/or rhynchosporium infection. However, like the area data, there was no correlation with the lesion severity data of the single interaction rhynchosporium DLA study.

Salt-Rhynchosporium Interaction

The experiment using selected lines subjected to salt stress, and how that caused differentiation of the symptoms of rhynchosporium, showed a strong increase in average lesion size when grown in salt-amended compost. This suggests that salt stress causes a predisposition to the biotic stress of rhynchosporium. This is not analogous to that seen in the Wiese *et al.* (2004) where decreases in powdery mildew infection with increased salt concentrations were seen. However, *Blumeria graminis* is a biotrophic pathogen which often shows opposite responses to those of necrotrophic pathogens, or hemibiotrophic pathogens in the necrotrophic feeding stage such as *R. commune* (Oliver and Ipcho*,* 2004; Glazebrook*,* 2005; Kliebenstein and Rowe*,* 2008; Mosher *et al.,* 2013). It has been shown that certain genes, such as Botrytis SUSCEPTIBLE 1 (BOS1) in Arabidopsis, are involved in both salt tolerance and necrotroph resistance, showing a connection between these stresses (Mengiste *et al.,* 2003; Atkinson and Urwin*,* 2012). It can also be seen that this is highly line/cultivar dependant, with a number of lines/cultivars, including the two elite cultivars used, being more susceptible under salt stress conditions. Two lines, however, showed a clear trend in the opposite direction, implying a tolerance to rhynchosporium gained from the salt stress. Both these lines, BW 902 and Aramir-M08, were selected from the complete collection due to their maintenance of dry weight in the landrace trials in 0, thus suggesting a difference in response mechanisms in reaction to increased salt levels that has an effect on rhynchosporium. This could be due to differences in the reaction to saline stress, as stresses such as salinity have been shown to cause cultivar-specific changes such as in the proteome of the shoots (Ramagopal*,* 1987) and the roots (Börner *et al.,* 2009) of barley. However, again it is seen that there is no correlation with lesion area in the single interaction rhynchosporium DLA study, nor the dry weight measurements in the single interaction salt compost study. The lesion severity data does not show a difference overall between the salt levels, but does when looking at the individual lines/cultivars. There is no obvious pattern to this (and, like the area data, there is no

correlations with the single interaction studies) but there is a large decrease in lesion severity for Aramir-M08 when grown in salt, supporting the conclusion from the lesion area data suggesting that there is a difference in response mechanism in this line.

Future Work and Implications

These results show the complexity of working with dual interactions that are highlighted in Suzuki *et al.* (2014), identifying that there is an overall trend only in the interaction of one abiotic stress (salinity) but not the other (Mn deficiency). It can be concluded that there seems to be no differences between the different sub-categories in how the stresses interact, with only individual lines having distinct differences. What this study has shown is that there are potential differences in these individual lines selected and how they interact with combined abiotic and biotic stresses. Specifically, identifying lines that show differences in the interaction with salt, and reduced lesion size in both Mn deficient and adequate systems. Further work is needed to validate the consistency of these variant line behaviours before mechanistic studies are pursued. Once fully validated, additional experimentation on the interaction of these stresses should also be done *in vivo* to assess the effects of the stresses occurring simultaneously. Other future trials could also build on the work by Pandya *et al.* (2005) that showed Mn supply reduced the stress caused by salt in barley seedlings, to gain an understanding in how this differs in the Bere lines that accumulate more Mn and that have greater Mn use efficiency. Once known, the differences in how these abiotic stresses interact with rhynchosporium can be further quantified. In addition, interactions of these abiotic stresses with other pathogens can be performed to assess the breadth of the effects on other biotic stresses, this was started in part with *P. teres* in subsequent work.

Identification and application of stress adaptation of the Scottish barley landrace Bere (*Hordeum vulgare* L.)

Identification of stress resistance/tolerance in the Bere barley population

Crop stress, both biotic and abiotic, causes large amounts of yield loss world-wide (Mittler, 2006; Paulitz and Steffenson, 2010). This loss of yield is highly variable, depending on certain environmental factors, ranging from low levels of stress causing minor percentages of loss to the extreme stresses that cause complete failure of the crop (Samarah, 2005), or complete economic loss in cases where it is impractical to harvest or no longer food safe. Preventative measures of stress, such as chemical control, can often take up a large amounts of money, time, and resources (Rai *et al.,* 2011) and do not completely prevent loss due to stress. Breeding for stress resistance is one effective method of preventing damage from biotic and abiotic stresses. However, current breeding programs currently focus of yielding in favourable conditions. This is reflected in the recommended lists for cultivars that is a useful guide when growing in reduced stress environments but is lacking in area that may be affected, potentially providing the users with unsuitable recommendations. With climate change these stresses will potentially occur in areas previously unaffected and could cause more frequent occurrences and/or more extreme levels (Ceccarelli *et al.,* 2010), highlighting the importance of robust crop breeding. Changes in environmental conditions will also change the habitable and prolific regions of different pathogens, causing a shift of pathogens to new areas, and increasing levels of disease in others (Seherm and Coakley, 2003; Duveiller *et al.,* 2007) with corresponding shifts away from other areas and reductions in susceptibility (Skelsey and Newton, 2015). This is likely to be exacerbated due to the mechanistic interconnectivity of the necrotrophic and biotrophic fungal defence in the plant systems, shifting pathogens to crops that have susceptibility inadvertently bred into them as a defence to pathogens of the other feeding mechanism (Glazebrook, 2005). This level of change is highly unpredictable when interactions are considered, for example different changes in the metabolome when multiple stresses are applied cause alternate changes in the resistance/susceptibility compared

to when the stresses are assessed independently (Newton *et al.,* 2012; Mikkelsen *et al.,* 2015a; Mikkelsen *et al.,* 2015b).

Landrace lines have consistently been shown to be a viable reservoir of genetic resistance to biotic and abiotic stresses in numerous different important crop species such as beans (Miklas *et al.,* 2003; Muñoz-Perea *et al.,* 2006), potato (Cabello *et al.,* 2013; Limantseva *et al.,* 2014; Pérez *et al.,* 2014), sweet potato (Gibson *et al.,* 2000; Gibson *et al.,* 2004), tomatoes (Agong *et al.,* 1997; Ji *et al.,* 2007; García-Martínez *et al.,* 2011; Moles *et al.,* 2016; Conicella *et al.,* 2017), and various different cereals (Blum and Sullivan*,* 1986; Arnason *et al.,* 1993; Xiao *et al.,* 2013). This is often found in the literature with barley landraces repeatedly showing an increased resistance or tolerance to a particular stress (Newton *et al.,* 2010). Examples of abiotic stresses include heat stress in Mediterranean landraces (Yahiaoui *et al.,* 2014; Cantalapiedra *et al.,* 2017), salinity stress in Syrian landraces (Weltzien and Fischbeck*,* 1990), and drought stress in the former two, Ethiopian and Jordanian landraces (Ceccarelli *et al.,* 1987; Al-Abdallat *et al.,* 2017; Abera*,* 2009). Examples of biotic stresses include: *Mlo* variants for resistance to mildew found in Ethiopian landraces (Jørgensen*,* 1992), multiple pathogens in Spanish landraces (Silvar *et al.,* 2010), and to the Ug99 stem rust race in Swiss landraces (Steffenson and Jin*,* 2006). A good source of resistance to biotic stresses are landraces in the local environment in which that stress is most prevalent, as landrace resistance to biotic stresses has been shown to be highly eco-geographically specific (Endresen *et al.,* 2011).

The Bere population tested in this thesis is a diverse set of lines of landraces collected from and grown in marginal lands across the highlands and islands of Scotland, having become adapted to many differing environments. This study has identified differences, within the Bere populations and between the Bere, other landrace lines and elite cultivars, looking at how they react to different stresses of both a biotic and abiotic nature. These studies revealed the clear advantage of particular landrace lines in tolerating particular stresses that affect elite cultivars. A portion of the resistance/tolerance to both the biotic and abiotic stresses was identified in genomic regions that do not have associated resistance/tolerance that are currently in use, suggesting that what was identified in these Bere lines were novel sources. This highlights the potential of the Bere population in breeding

for biotic and abiotic stresses. This potential is thought to be caused by the diversity in the marginal environmental conditions between and within the different highland and island areas that Bere barley has been selected on and become adapted to. The current population of landraces held in germplasm collections is much reduced due to the social-economic changes in the 20th century causing the replacement of traditional landrace lines with genetically narrow elite cultivars (Negri *et al.,* 2009; Dwivedi *et al.,* 2016). Whilst this reduction is an important loss, there is still a large potential for landraces to be used due to the increase level of diversity compared to the elite breeding population (Newton *et al.,* 2010; Langridge and Waugh, 2019; Monteagudo *et al.,* 2019).

The reason for selecting the three stresses tested in this thesis was due to a combination of different factors. One selecting factor was the variability of these stresses in the geographical region in which the Bere barley grows. The islands of Scotland have regions that are affected by salt due to year-round salt-laden winds (Dry and Robertson, 1982), and have highly alkaline soils that show Mn deficiency (Martin *et al.,* 2008b), with Bere lines having been shown to have improved growth on the latter (George *et al.,* 2014; Schmidt *et al.,* 2018). Similarly, one of the selecting factors of rhynchosporium stress was the anecdotal evidence of reduced infection of *R. commune* in Bere lines from interviews with farmers by Mahon *et al.* (2016). A secondary selection factor was the importance of these stresses in a Northern European, and/or a global context. Figure 33 shows that all three stresses have large economic importance over at least four different continents. It can be seen also that two economically influential countries, USA and Australia, are impacted by all three stresses, Australia having all stresses occurring in overlapping regions. Worldwide it can be seen that salt stress affects the widest ranging of regions, with all habitable continents bar Europe being directly affected. Within the Northern European context, the rhynchosporium and Mn-deficiency stresses are the most important of those tested. The UK has regions that are affected by both, with regions overlapping. Similarly, this is shown for Denmark.

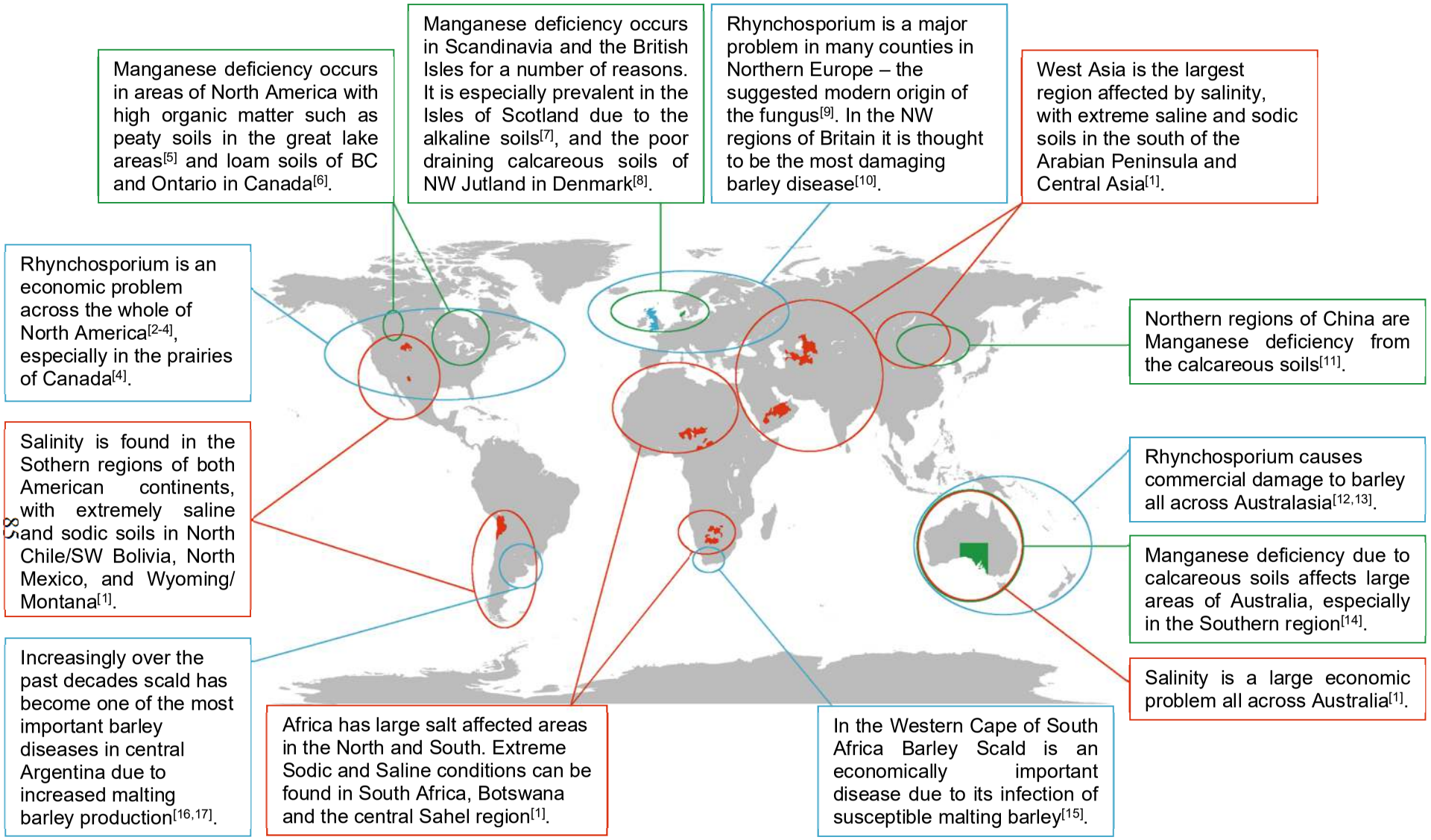


Figure 33) A world map highlighting key regions affected by the three stresses investigated in this thesis; salinity (**Red**), manganese deficiency (**Green**), and rhynchosporium (**Blue**). Regions that have particularity high levels of one stress are coloured in the corresponding colour. References numbered as 1) Wicke *et al.* (2011), 2) Caldwell (1937), 3) Webster (1980), 4) Tekauz (1991), 5) Adriano (2001), 6) Reid and Webster (1969), 7) Goldberg *et al.* (1983), 8) Steenbjerg (1935), 9) Brunner *et al.* (2007), 10) Henley (2015), 11) Tong *et al.* (1997), 12) Murray and Brennan (2010), 13) Cromey (1987), 14) Graham *et al.* (1982), 15) Robbertse *et al.* (2000), 16) Carmona *et al.* (1997), and 17) Ríos *et al.* (2007).

Manganese use efficiency

The breeding of Mn use efficiency will allow increased production of areas with Mn deficiency that is a common problem in cereals across the UK (Neilsen *et al.,* 1992; Chalmers *et al.,* 1999) and particularly in certain soils of the Scottish Isles (Dry and Robertson*,* 1982; Scholten *et al.,* 2004; Dry*,* 2016), as well as areas around the world with major issues in Southern Australia (Graham *et al.,* 1982).

Manganese deficiency tolerance showed the clearest evidence of a mechanism of use efficiency common to the majority of the Bere lines. It could clearly be seen that the Bere lines, as a whole, were able to better retain the maximum quantum yield of photosynthesis under Mn deficiency indicating better Mn use efficiency, whilst the elite lines showed large effects of Mn deficiency. This is supported by the mineral concentration results that showed that the Bere lines had significantly greater Mn content in their shoots under Mn deficient conditions. This allowed for identification of individual Bere lines that were the most Mn use efficient lines tested, including: Bere 24268 A 71, Bere 45 A 23, and Bere 47 A 25. Genome-wide association analysis also enabled the identification of many genes that could be associated with increased Mn acquisition and utilisation. These included promising candidates that encoded for proteins such as a MATE efflux family protein and a Nramp1 metal transporter, both of which have been associated with increased Mn acquisition and related tolerances to other nutrient deficiencies (Rogers and Guerinot*,* 2002; Cailliatte *et al.,* 2010; Ishimaru *et al.,* 2012; Castaings *et al.,* 2016; Wu *et al.,* 2016).

Mapping of the currently identified Mn efficiency locus (*Mel1*) from a Japanese cultivar has identified the position to a 1.4 cM region on 4HS and confirmed the role in Mn use efficiency (Pallotta *et al.,* 2000; McDonald *et al.,* 2001; Pallotta *et al.,* 2003), allowing it to be used in breeding programmes to breed for Mn use efficient elite cultivars. Similar work could be performed using the chromosomal region in 2HL identified in this study and in Lloyd (2000), to provide additional targets for the breeding programmes and to possibly compliment the use efficiency traits provided by *Mel1*. Allowing for greater use efficiency will allow for greater yields on these marginal lands, as well as reduced need for the use of costly Mn foliar supplements (White and Greenwood*,* 2013; Leplat *et al.,* 2016).

Salinity tolerance

The breeding of salt tolerance is similarly important with an estimated 6.5% of land globally being salt-affected (FAO, 2015). This is expected to increase with the use of salt contaminated water for irrigation and the change in climate (Ayars *et al.,* 1993; Umali, 1993; Rengasamy, 2006; Wei *et al.,* 2018).

When assessing salt tolerance with regards to biomass accumulation under salt stress the Bere lines were, on average, no more tolerant than the other landrace lines. However, it was apparent that the rate of biomass reduction with increasing salt stress in the elite cultivars was, on average, double that of the landrace lines including the Bere lines. This shows the need for increased salt tolerance in the elite cultivars as well as the potential of landrace lines to provide that tolerance. Salt tolerant individuals that increased biomass with increasing salt levels were identified, including: Prize Prolific-196, Bere-118, and Bere 49 A 27 Shetland. The GWAS identified a region associated with maintained biomass in increasing salt conditions on chromosome 5HL, along with an additional marker on 3HL. Within these associated regions there were five candidate genes that encoded for proteins associated with increased salt resistance, including an Acyl-CoA-binding domain-containing protein which is associated with an increased drought tolerance (Raboanatahiry *et al.,* 2015; Du *et al.,* 2016).

Due to the scale of the problem of salinity, much research has been undertaken to study its tolerance, hence a number of associated loci have been identified in barley (Miyazaki *et al.,* 2010; Siahsar and Narouei, 2010; Shavrukov *et al.,* 2010; Xue *et al.,* 2010; Zhou *et al.,* 2012a; Fan *et al.,* 2016; Saade *et al.,* 2016; Xue *et al.,* 2017). This includes the region on chromosome 5H identified in this study, but not that which was identified on chromosome 3H. Within these, the gene HKT1;5 in barley has been located to a 0.5 Mb region, characterised and identified for potential use in breeding programmes (Hazzouri *et al.,* 2018), similar processes could be used to refine and incorporate the regions identified in this study. The mechanisms of salt-stress are complex, with multiple different effects, hence the mechanisms of tolerance need to be varied, identifying the need for multiple loci associated with a superior trait. Allowing for greater salt tolerance in elite cultivars will increase the yield in marginal lands with reduced quality water resources, and will help maintain yields in areas where salt stress will or might increase (Ashraf and Foolad, 2013).

Rhynchosporium resistance

Development of rhynchosporium resistant cultivars is also of benefit as it is considered the major economic barley disease in the UK (Fitt *et al.,* 2012; Havis *et al.,* 2015) and amongst the most important worldwide (Zhan *et al.,* 2008). Currently there are a number of R-genes that provide resistance to rhynchosporium (Zhan *et al.,* 2008), but due to the pathogens ability to readily remove or modify certain targeted effectors there is a constant need for new genes to replace them and to bolster the resistance (Avrova and Knogge, 2012).

Trials that inoculated lines with rhynchosporium showed that with some isolates the Bere lines had greater resistance than the other landraces and the elite cultivars. This is further supported by the field trials that showed the four-fold relative average increase in observed disease in the elite cultivars compared to the Bere lines, showing the need for increased resistance in the elite populations and the potential of the Bere lines to provide this. From these studies, lines including Bere 45 A 23, Bere 58 A 36 Eday, and Bere 8-125 were consistently disease resistant in both field trials and the DLA experiments, thus eliminating any resistance due to unusable structural changes such as increased height. Analysis with the genotypic data identified regions in both data sets that had not previously been identified, including a region on chromosome 5H that previously has not been associated with a Rrs gene. This suggests unique methods of disease limitation that could be identified and used in breeding programmes. A number of genes with putative functions associated with disease resistance were identified in these regions, such as the aptly named disease resistance proteins and proteins with anti-fungal domains such as the Gnk2 homologue. Identification of the gene(s) associated with the reduced disease should allow the transfer into elite backgrounds and provide potential.

Development of lines that are resistant to rhynchosporium would help alleviate some of the estimated £7.2 million worth of losses, as well as reducing the need for fungicide treatments (Avrova and Knogge, 2012; Paveley *et al.,* 2016). Additionally, these genes may have further implications for other pathogens, such as accounting for the reduced foliar disease associated with Bere lines observed by farmers in Orkney (Mahon *et al.,* 2016), or an associated negative effect on biotrophic or necrotrophic pathogens due to the common inverse relationship between the two lifestyles (Glazebrook, 2005).

Reaction to dual stresses

Further complications with regards to the changing climate would be the increased incidence of interaction of the stresses, which have been rarely studied. Examples of this would be the negative interaction between saline and drought stresses (Ahmed *et al.,* 2013), or the inverse relationship between biotic and abiotic stresses in a stress response gene in rice (Xiong and Yang*,* 2003) and barley (Ali *et al.,* 2014) . Though, conversely, examples of positive stress interactions have been shown, as in the examples from Wiese *et al.* (2004) that showed an enhanced resistance of barley to powdery mildew when exposed to abiotic stresses such as salinity. The interactions between the stresses used in this study were selected due to the occurrence of all three in the regions where the Bere lines originate from. Thus, it was thought that these lines could show resistance mechanisms that better accommodate for the interaction of these stresses.

Dual interaction studies from this thesis highlighted the complexity of working with and measuring multiple stresses in conjunction. Of the two abiotic stresses tested with rhynchosporium, only high salt levels caused a consistent change to the infection level, with the added salt stress causing an increase in rhynchosporium lesion area. Differences were seen within lines/cultivars for both salt stress and manganese deficiency, but with no correlation with each other or with previous data (or with stress measurements for the Mn), possibly indicating independent mechanisms of resistance/susceptibility. However, this allowed for identification of lines that performed differentially with stress, including lines that went against the trend and performed better with salt stress.

Identifying the interaction of different stresses has been pursued in this thesis, showing the differences in the interaction of biotic and abiotic stresses between different lines, the importance of which has been identified in multiple studies (Atkinson and Urwin*,* 2012; Suzuki *et al.,* 2014; Pandey *et al.,* 2017). However, it should be noted that the study of dual interactions is incomplete as additional stresses could change the metabolome profile of the plant giving a different response to that seen in dual interactions (Mikkelsen *et al.,* 2015a; Mikkelsen *et al.,* 2015b).

Role of Bere barley in breeding for climate change

Breeding for stress resistance is of particular importance due to climate change causing a change in interrelated stresses. Developing crops that are protected against climate change due to robust defence to increased stress is critical for future agricultural sustainability.

One commonly referenced example is that climate change is expected to increase the areas of salinity and drought in future by causing weather patterns to shift and raising water tables (Wang *et al.,* 2003; Munns and Gilliham, 2015). Salinity, in particular, is becoming an increasing problem with the irrigation of land using brackish water under drought conditions (Umali, 1993; Ayars *et al.,* 1993; Wei *et al.,* 2018). Additionally increasing dryland salinity is becoming a problem due to sea level rise causing elevated saline groundwater tables thus affecting a large amount of coastal lands that have previously been unaffected (Rengasamy, 2006), which could further be affected by rising water tables due to deforestation in temperate zones (Sahagian, 2000). Breeding for tolerance of micronutrient deficiencies and toxicities, such as Mn use efficiency, is crucial as shifts in temperature and rain patterns may make growing barley on more marginal lands an option or necessity (Morton *et al.,* 2015; Mizyed, 2009; Berglund, 2003). Climate change induced changes in the rhizosphere can cause additional problems, such as warming reducing the heterotrophy and water-availability reducing the soil respiration, that will affect the availability of Mn and other micronutrients (Rengel, 2011; Rengel, 2015). Finally this is of greater importance with regards to climate change as the *Rhynchosporium commune* fungus is highly adaptive under stress and could spread with changing climates (Stefansson *et al.,* 2013), or become more severe in different seasons (Newton *et al.,* 2008).

Changes in one stress due to climate change could result in an unpredicted change in the level of another separate stress (Prasad *et al.,* 2011; Ramegowda and Senthil-Kumar, 2015; Pandey *et al.,* 2017). Breeding for increased resistance will allow for protection from unpredictable stress increases as a result of changes in an interacting stresses, giving an improved yield stability for changing environments (Mickelbart *et al.,* 2015). This highlights the importance of breeding climate change resistant crops to be able to maintain yield overall and on affected lands.

As shown, landraces play a potentially important role in the development of crops that are able to withstand stress, thus they could play a pivotal role in the development of crop that are able to withstand their combined effects. The work from this thesis has shown the potential of the Bere barley landrace to provide some of the resistance traits needed to be able to develop these robust crops (Table 9). This will help allow for the better maintenance of yield in areas of land quality deterioration and areas in which pathogenicity increases, as well as allowing the growth on current marginal lands that will help protect and increase global production. Additionally, certain lines may contribute a general resistance to a number of different stresses due to the interconnectivity of the stresses through shared physiological and molecular responses that are found over a number of stress responses (Pastori and Foyer, 2002; Mithöfer *et al.,* 2004; Fujita *et al.,* 2006; George *et al.,* 2017). Of the lines identified in this thesis (Table 9) most only perform well in one of the stresses measured. The exception to this was line Bere 45 A 23 that showed increased Mn use efficiency as well as increased rhynchosporium resistance, indicating possible mechanisms of a general or connected stress resistance. Similarly, Bere 47 A 25 showed moderate to high stress resistance in the two stresses tested, but there were no results for rhynchosporium due to limited seed stock. However, as noted above, interactions of stresses can be unpredictable, especially when in conjunction with more than two due to differing reactions in the metabolome (Newton *et al.,* 2012; Mikkelsen *et al.,* 2015a; Mikkelsen *et al.,* 2015b). Some of the lines were included in the dual interaction study but none were identified as having a unique interaction.

Table 9) The nine landrace lines that have been identified as a good source of stress resistance to one of the stresses tested, along with information on the performance of those lines, in relation to the other lines tested in the screen, when the other stresses were applied.

Landrace Lines	Manganese Use Efficiency	Salinity Tolerance	Rhynchosporium Resistance
Bere 24268 A 71	High	Med	Med
Bere 45 A 23	High	Med	High
Bere 47 A 25	High	Med-High	Not Screened
Bere 49 A 27 Shetland	Med-High	High	Med-Low
Bere 58 A 36 Eday	Med-High	Med	High
Bere 59 A 37 Uist	High	Med	Med-High
Bere 8-125	Low	Not Screened	High
Bere-118	Med-Low	High	Med-Low
Prize Prolific-196	Med	High	Low

Challenges associated with breeding for stress resistance in barley

As shown, breeding for resistance to stresses will aid in the protection of crop yields, as climates change and become more unpredictable, by increasing the robustness of the cropping system (Ceccarelli *et al.,* 2010). Stress resistant cultivars will also increase future production by making previously unusable lands productive and increase sustainability of yield on more marginal lands. Additionally, these cultivars would help create more sustainable agriculture due to the reduced need for preventative measures such as fungicide and nutrient additions that are both costly and potentially damaging to the environment (Humphreys, 2007; Witcombe *et al.,* 2008).

Once genetic regions of interest have been identified in landraces of crops such as Bere barley, the main challenge is the integration of these traits into an elite background. This is highlighted well with multi-gene traits such as salt tolerance that has had little success in integration via conventional breeding programmes (Flowers and Flowers, 2005), though this is likely to improve with advancements in genetics (Ismail and Horie, 2017) as has been seen with rice (Negrão *et al.,* 2011) and wheat, but with few examples in barley (Shahbaz and Ashraf, 2013). For simpler traits this is easier, but there are still challenges when crossing non-adapted germplasm such as landraces. One challenge is that often the region of interest is closely linked with traits that can have a negative impact on the plants and reduce the quality or yield by linkage drag. This makes these undesirable traits more likely to be incorporated into the elite plant along with the desired trait as they are harder to separate, resulting in poorly performing crops that are not economically competitive (Muñoz-Amatriaín *et al.,* 2014) . Additionally, the genes of interest could be within a region with little to no cross-over, such as the pericentromeric regions, making transfer from the landrace background using traditional breeding practices unfeasible (Künzel *et al.,* 2000; Mascher *et al.,* 2017). To aid in the breaking of these linked regions the steps to identify the causal gene can be undertaken. This can be done by first fine mapping to reduce the targeted location by using homozygous descendants, of crosses between the resistant/tolerant line and a susceptible elite cultivar, that are fixed for known regions of resistance/tolerance and segregating in the region of interest. By comparing the resistant/tolerant

lines to the near isogenic lines (NILs) using a higher concentration of markers in the smaller region it is possible to isolate the region of interest further. Coupled with RNA sequencing, to identify which genes are differentially expressed, the candidate genes can be significantly reduced to a number able to identify the causal gene through sequencing (Liller *et al.,* 2017).

Before introgression into the elite lines there may be further problems associated with the identified regions, including negative aspects inherent in the trait of interest. An example of this would be in disease resistance, as the mechanisms of resistance would need to be separated from similar mechanisms of disease avoidance and disease tolerance, all of which would cause a reduction in disease. One method of avoidance would be from genes associated with plant height in rhynchosporium reduction. These are identified as rhynchosporium reduction genes due to the associated increase in disease escape via the decreased transmission of spores through splash dispersal (Zhan *et al.,* 2008), thus avoiding the disease. These traits are unusable as the inherent negative trait, such as increased height diverting resources away from yield and increasing lodging, cannot be broken as it is the cause of the reduced disease, and cannot be incorporated as it would heavily reduce the economic effectiveness of the elite cultivars. Additionally, traits can come with associated costs with the mechanisms of stress resistance/tolerance due to the diversion of energy into the implementation of the effect, resulting in a yield penalty. This is commonly seen with disease resistance (Brown*,* 2002; Walters and Boyle*,* 2005; Ning and Wang*,* 2018), but also with abiotic stresses (Wilson*,* 1988; Knight and Knight*,* 2001; Minhas *et al.,* 2017).

Future breeding methods, outlined below, could be implemented to incorporate the identified genes, allowing easier breaking of linked undesirable genes from non-adapted germplasm. These could include transgenic approaches to transfer genes involved in stress resistance into the elite host, or through modification of the elite host gene to replicate the differences found in the landraces, using technologies such as the CRISPR/Cas9 system (Bhatnagar-Mathur *et al.,* 2008; Zhang *et al.,* 2014). Whilst this does not account for changes that may occur in the interaction of such genes in the elite background, it does hold potential to make transfer of most traits more rapid.

Further work

These studies identify that in all three of the stresses tested the Bere group, as a whole or individual Bere lines, have a superior resistance/tolerance. Due to the diversity of the different environments where Bere lines grow and have become adapted, and the diverse range in the levels of stress adaption shown in the Bere lines for the stresses tested, such germplasm resource potentially holds resistant traits to a number of other different stresses that could be tested for. This testing can be aided by the number of existing crosses that are available between Bere lines and elite cultivars.

Only one pathogen (but multiple isolates) was tested in this study, *R. commune*, a hemibiotrophic foliar fungus. Therefore, a compliment of other foliar fungi could be tested to see the effect of different interactions in Bere lines, and this has been started in a study with the use of the necrotrophic leaf fungi *Pyrenophora teres*. Results from this, whilst incomplete, indicate intermediate resistance in some Bere lines, including one found to also be resistant to rhynchosporium. Similarly, resistance to *P. teres* has been identified in Turkish landraces (Çelik Oğuz *et al.,* 2017), though the related hemibiotrophic fungi *Pyrenophora graminea* showed high levels of susceptibility in a Bere line, but only one line was tested (Cockerell*,* 2002). Biotrophic foliar fungi should also be tested on the Bere landraces, though anecdotal evidence suggest that Bere barley in general is susceptible to powdery mildew infection (Wright *et al.,* 2002). In addition to foliar fungal pathogens, fungi that infect the roots should also be assessed such as the hemibiotrophic fungus *Cochliobolus sativus*. This affects both root and shoot tissue, causing the diseases common root rot and spot blotch, respectively, and has been shown by Whittle (1977) to have reduced seedling infection in a Bere line compared to elite cultivars. Resistance to the root disease take-all by the fungal agent *Gaeumannomyces graminis* may also be of interest to screen for in the Bere collection due to the positive association of Mn use efficiency and resistance in wheat (Wilhelm *et al.,* 1988), though this connection was unable to be identified in barley (Lloyd*,* 2000).

Other pathogens could also be tested in this Bere population, identifying additional sources of resistance to diseases such as to barley yellow mosaic virus in a Chinese landrace (Konishi *et al.,*

1997), to barley yellow dwarf virus and Barley mild mosaic virus in Spanish landraces (Silvar *et al.,* 2010), bacterial leaf streak in wheat landraces (Adhikari *et al.,* 2012), cereal cyst nematodes from a landrace derived Australian cultivar (Barr *et al.,* 1998), and resistance to greenbug (*Schizaphis graminum*) in a Korean winter barley landrace (Porter *et al.,* 1998).

Large differences in the soil pH between the different environments where Bere lines grow, suggest a large difference in micronutrient accumulation ability. This is further suggested in the mineral analysis data from the Mn study that indicates greater levels of accumulation of calcium, copper, and phosphorus in Bere lines compared with elite cultivars, as well as a number of Bere lines that accumulate increased levels of zinc verses the elite cultivars. The accumulation of calcium and phosphorus is of particular interest as alkaline soils cause a reaction of these two elements making the latter unavailable to the crop (Hopkins and Ellsworth*,* 2005; Shen *et al.,* 2011). Additionally, Bere lines that were not included in this analysis may show other differences due to the different location of origin. Lines adapted to more acidic environments would not perform well in Mn deficient environments, but may have large differences in phosphorus, iron, and aluminium (Ladouceur *et al.,* 2006; Zheng*,* 2010) such as the Al-toxicity/low pH tolerance association found in one Bere line by Stølen and Andersen (1978).

Other abiotic stresses should also be explored, in particular drought stress. This study identified a number of Bere lines that are salt tolerant, which is often associated with a tolerance to drought (Golldack *et al.,* 2014; Zhu*,* 2002), and thus may have increased drought tolerance. However, Brown (2017) shows that Scotland has high levels of soil wetness vulnerability, especially in the highlands and islands where Bere grows, indicating that these soils are more prone to waterlogging (Lilly and Matthews*,* 1994). Traits related to improved waterlogging tolerance, such as reduced leaf chlorosis, are important as barley is one of the most susceptible cereal crops to this stress, and can be screened in a similar manner to that for Mn use-efficiency undertaken in this study (Bertholdsson*,* 2013), allowing for whole plant stage screening that has been shown to be a more effective indicator (Setter and Waters*,* 2003).

Concluding remarks

Together these results show the positive traits of stress resistance/tolerance in the Bere barley collection, along with other landrace lines, and the potential that they have in breeding for these stresses, as well as identifying that there is a large possibility of potential resistance/tolerance in other stresses. This would be further amplified if one of these traits was a general resistance/tolerance to multiple stresses, this would increase the breeding potential of this trait due to the reduced complexity compared with the need to incorporate multiple traits to provide the same benefit, thus widening the potential marketability.

This thesis has also highlighted the implications of how these stress resistance/tolerance traits will benefit breeding for crops with improved robustness for improving yield on marginal lands, protect from minor and sudden stresses, reduce the need for chemical input to increase sustainability in farming, and protect future crops from the changing environment caused by climate change. But the thesis has further highlighted the complexities involved when assessing the impact of interacting stress and plants response to that.

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