Relationship of Grain Anthocyanins with Winter Hardiness in Rye (*Secale cereale L.)*

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ABSTRACT

Many Canadian varieties of autumn seeded rye (*Secale cereale* L.) demonstrate very high winter hardiness and have much higher winter field survival (WFS) scores than other cereal crops such as wheat and barley. This high frost tolerance for rye was in a previous study found to be associated with the production of glycosylated cyanidins in crown and leaf tissue during cold acclimation in the autumn. Anthocyanins and other flavonoid compounds are also present in the dark rye grain; thus, the possibility that WFS levels could be predicted from specific grain extractable compounds was investigated in this study. To produce grain for the analysis, 96 rye cultons with determined WFS scores were grown in a greenhouse environment (2020-2021) and under field conditions (2021-2022). The greenhouse population developed overall taller plants with longer spikes and awns as compared to the field plants. However, only the spike length of greenhouse plants showed a strong positive association with WFS (p<0.001; R^2 = 0.386), whereas the seed protein concentrations was negatively associated (p<0.001; R^2 = -0.206). For field plants, the germination frequency, plant height, and traits contributing to larger seeds (seed length, width, weight) were positively associated with WFS (p<0.001; R^2 = 0.255 to 0.615), and similar to greenhouse grain, the seed protein concentration was negatively correlated (p<0.001; R^2 = -0.309). These associations supported cultons developing large seeds with low protein concentration should be selected for WFS studies as these lines are more likely to produce a high germination frequency and have a high WFS. Determination of anthocyanin concentration in greenhouse grain revealed a wide distribution (0.9 to 10.7 µg mL^{-1}) and a mean value of 3.1±1.6 µg, mL^{-1}. A similar mean was seen for field grain (3.01 ± 1.0 µg mL^{-1}) having concentrations ranging from 0.72 to 5.8 µg mL^{-1}. Analysis of the grain extracts by high performance liquid chromatography (HPLC) and QTOF-MS analysis revealed nine identifiable anthocyanin compounds, which was fewer than the 18 identified for leaf and crown tissues. However, it was not possible to draw any firm conclusions regarding the association of grain anthocyanin concentration or specific anthocyanins with WFS scores. A large number of minor anthocyanins like compounds in the grains remained uncharacterized and a more detailed study on these is needed to definitely determine if any relationship exists between grain constituents and WFS

scores. These studies can also add to the health benefits flavonoid compounds provide to rye products.

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Chapter 1. Introduction

1.1. Winter hardiness in rye

Rye (*Secale cereale* L.) is a grass from the Triticeae tribe of the Pooideae subfamily and is a member of the cereal family which includes Canadian staple crops such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and oat (*Avena sativa*). In contrast to these crops, rye has a strong self-incompatibility system (Lundqvist, 1954; Schlegel, 2014); thus, it reproduces via cross-pollination, where multiple rye plants pollinate each other via wind-borne pollen. The rye cultivars can be divided into two broad categories based upon their growth habit. Spring rye grows and matures rapidly after spring seeding and produces grain in the autumn. Winter rye is seeded in the autumn and experiences a lengthier growth period when it remains small and leafy before over-wintering in a state of low energy activity. When days are becoming longer in the early spring, winter rye regrows from the crown to produce mature plants and grain in the late summer. The winter rye's ability to survive frost during winter is acquired through four to seven weeks of cold acclimation and vernalization processes (Thomashow, 1999), which are initiated in plant tissues by low temperatures and changing light conditions in the autumn (Franklin and Whitelam, 2007; Legris *et al.*, 2017). These activities stimulate many developmental and metabolic pathways that contribute to accumulation of freezing tolerance in plants (Winfield *et al.*, 2010). The vernalization process in particular gives the shoot apical meristem the competence to transition to reproductive growth when long-day conditions appear in the spring (Winfield *et al.*, 2009).

One notable aspect for many rye varieties is the high tolerance to a variety of abiotic stress factors including drought, nutrient deficiencies, and cold (Schlegel, 2014). Rye has one of the highest drought tolerances among cereal crops (Schittenhelm *et al*., 2014; Hlavinka *et al*., 2009) and can be grown in low-fertile soils; this allowed the early rye farmers to cultivate rye under sub-optimal conditions, yet still producing an acceptable yield (Parat *et al.*, 2016). Cold tolerance, also known as winter hardiness and quantified by winter field survival (WFS) scores is another important environmental tolerance for rye

(Fowler *et al.*, 2014). This trait is especially high in winter types, some of which are the most winter tolerant cereal crops (Fowler and Limin, 1987). The specific criteria for winter hardiness in a plant can vary depending on different injury-causing stressors that occur during winter, though resistance to frost damage usually plays a major role (Würschum *et al.*, 2017). The frost tolerance of rye acquired during cold acclimation in the autumn is one of the highest among cereals, which enables winter types to tolerate low freezing conditions for extended periods of time (Fowler *et al.*, 2014).

The high cold and freezing tolerances of rye make this crop a valuable resource for research into the underlying mechanisms of WFS in cereal crops. By studying and improving the overall understanding of how these crops develop their resilience to stress inducing environmental conditions, it may be possible to develop crops more capable of thriving in colder climates.

1.2. Impact of low temperature exposure

One of the issues that low temperatures cause in plants is the disruption of the photosynthesis process (Öquist *et al.*, 1993). Lowered temperatures decrease the rate of enzymatic reactions, increases the viscosity of the cell membranes, and slows down the movement of electrons through them. This disrupts a number of cellular processes, including the efficiency of the mechanisms behind photosynthesis such as photochemical efficiency and electron transport capacity (Ensminger *et al.*, 2006; Hüner *et al.*, 2012; Paredes and Quiles, 2015; Hajihashemi *et al.*, 2018). Interruptions of these vital processes can present a number of different issues for a plant, including being a source of injury.

The disruption of photosynthesis and other damages caused from exposure to low temperatures, can vary depending upon the severity of the exposure, the plant species, and other factors. Tropical crops such as peppers or papaya can suffer significant loss in yield quality when subjected to low above zero temperature (chilling) stress, displaying symptoms such as softened surfaces on the produce, scalded skin, and increased vulnerability to disease (Paull, 1990). Even among those crops able to survive low

temperatures, not all of them experience chilling injury to the same degree though. Winter-adapted crops such as winter rye have a greater tolerance of environmental low temperatures below zero, being able to survive, and even remain partially active during the winter.

The high winter hardiness of winter rye allows it to perform better than other cereals in cooler, higher latitude regions. This quality makes rye a potentially valuable crop for nations with a cooler climate like Canada. As compared to Northern, Eastern, and Central Europe, rye is a minor crop in Canada. In 2020, rye was seeded over 100,000 hectares in Canada to produce 320 thousand metric tons, contributing 2.6% of global rye production (USDA, 2021). In Canada, the low ambient temperatures along with wind chill factors during winter make adaptations aimed at addressing these factors necessary for any crop to be successful. Some adaptations can be behavioral in nature, such as winter dormancy, while others may focus on the chemical composition and metabolism of the plant's tissues, such as the process of undergoing cold acclimation in the fall in preparation for winter. The latter may arise inherently in the developmental stages of the plant or due to the expression of abiotic stress related transcription factors such as HSP, MYB, bHLH, NAC, bZIP, C2H2 and CBF factors, which become up-regulated in plants experiencing cold stress (Thomashow *et al.*, 2001; Chinnusamy *et al.*, 2007; Kong *et al.*, 2020). Some examples of a metabolic change in a cold-stressed plant may include: changes in soluble protein content (Thomashow, 1999), accumulation of apo-plastic antifreeze proteins (Griffith and Yaish, 2004), increased production and accumulation of soluble sugars (Halford *et al.*, 2011; Tarkowski and Van den Ende, 2015), accumulation of antioxidants (Cook *et al.*, 2004) and alterations in the stability of the plasma membrane (Lindén *et al.*, 1999; Uemura *et al.*, 2006; Ruelland and Collin, 2012). One potential method to estimate levels of winter hardiness exhibited by winter rye would be to measure the concentrations of cold tolerance predictive chemical compounds in the plant's tissues. One example of such a compound would be flavonoids such as anthocyanins, of which certain cyanins have a relationship with winter hardiness in winter rye (Bahrani *et al.*, 2019) and frost tolerance in Arabidopsis (Schulz *et al.*, 2016).

1.3. The role of anthocyanins for winter hardiness

Many secondary metabolites such as flavonoids play important roles in the development and growth of plants (Taylor and Grotewold, 2005; Agati *et al.*, 2012). One example would be responses to stress conditions including nutrient deficiencies, temperature extremes, and high light intensities (Schmitz-Hoerner and Weissenböck, 2003; Khlestkina, 2013; Schulz *et al.*, 2016) (Olenichenko *et al*., 2006; Zakhleniuk *et al.*, 2001). The presence of flavonoids enhances the plants individual tolerance to low temperatures, along with aiding the adaptive responses towards abiotic stress induced by low temperatures (Agati *et al.*, 2013; Kong *et al.*, 2020). Flavonoid compounds have been observed as significant contributors towards antioxidant protection in winter hardened cereals, with anthocyanins specifically being more concentrated in cultivars with high frost resistance (Kolupaev *et al*., 2020).

The relationship between winter hardiness and anthocyanin compounds in rye has been determined to a certain extent. Increased concentrations of anthocyanins and other flavonoids are observed in cereal seedlings experiencing cold hardening and to a greater degree in plants able to develop high winter hardiness (Kolupaev *et al.*, 2020). For rye, it has been demonstrated that the presence of certain cyanin compounds in cold-acclimated leaves and crown tissues is an indicator of high levels of accumulated winter hardiness (Bahrani *et al.*, 2019). This is a highly valuable discovery, as it would allow for more efficient assessment of the winter hardiness in rye lines along with improving understanding of the cold and freezing tolerance mechanisms in winter rye (Bahrani *et al.*, 2019). The next step for research in rye winter hardiness therefore, would be to see if a similar relationship between certain anthocyanins and WFS can be demonstrated for the rye grain. If a rye grain has anthocyanin compounds that correlate with the plant's tolerance to cold, then that would be a viable information for the selection of winter-hardy genotypes. The ability to predict the winter hardiness of a rye genotype from grain material would support breeding efforts by reducing the number of lines to grow for winter survival testing. Instead

the anthocyanin or other compounds in rye grain could be extracted and analyzed for estimation of genotype's winter condition performance. This could save time and expense, streamlining the development process for breeders as well as improving the producer's means of predicting the performance of a crop.

1.4. Hypothesis and objectives

1.4.1. Hypothesis

Previous research on winter rye has determined that there is a significant relationship between winter hardiness and the cyanin group of anthocyanins in the crown and leaf tissue of cold-acclimated winter rye cultivars (Bahrani *et al.*, 2019). For this study, the null hypothesis is that there is no significant correlation between the anthocyanin content in winter rye grain and winter hardiness in over-wintering plants. Expanding on this field of study, the intention of this work is to determine if specific anthocyanins or other extractable compounds in grain can be associated with a genotype's WFS score.

1.4.2. Objectives

The objectives for this study are:

- Determine the total content and type of grain anthocyanins in a rye germplasm panel with varying degree of cold hardiness.
- Assess the relationship between grain anthocyanin content and winter-hardiness in winter rye.
- Assess the relationship between grain anthocyanin content and grain morphology in winter rye.
- Identify if any grain-specific extractable compound can be correlated with winter hardiness or grain size in rye.

Chapter 2

Literature Review

2.1.1. The history of rye cultivation

Rye was first domesticated in the Fertile Crescent during the Neolithic period (10,000 BC) and has been a major cereal crop in Europe since the Roman Empire era (100 BC). It was most intensively cultivated during the high Middle Ages, when the increasing population density of cities gave rise to a high demand for agricultural products (Behre, 1992). The naturally high tolerance to drought and nutrient-poor soils has made it possible to cultivate rye in soils with poor water retention, such as sandy or infertile soil, that is unsuitable to most crops (Schittenhelm *et al*., 2014; Hlavinka *et al*., 2009). Due to the great frost tolerance, winter types of rye have long been cultivated in Scandinavian countries and Russia and also been widely spread to Germany and Poland. While the prominence of rye has been declining in European agriculture, it is still a notable crop in the current day and age. The most productive rye growing year in recent history was 1990, where over 35 million tons were harvested in Europe. In the past couple of decades however, those numbers have been in decline, due to a variety of economic and cultural factors (FAO Stat, 2022). In 2018, over nine million tons were harvested throughout Europe, constituting 89% of the global rye production. This production largely takes place in Russia, Germany, and Poland, though other nations such as Belarus and Ukraine were noted to have harvests totaling in the hundreds of thousands of tons as well (FAO Stat, 2022).

Rye was not cultivated in North America until around the 16th and 17th centuries, when it was imported along with other European crops by European settlers (Bushuk, 1976). Preserved records that contain mentions of the introduction and selection for different lines of rye in Canada as a nation date back to the 1890's, where the breeding and development of several rye lines were mentioned in reports of the Dominion cerealist (Mackay, 1891). Rye breeding efforts then dropped off for a period of time in favor of other cereals, until resurgence in interest around 1940. New lines of rye were developed by several institutions, including the University of Alberta, and the University of

Saskatchewan. This increase in breeding and developer efforts for rye would continue until the late 1980's, when the demand for the crop began to decrease. Reasons for this decline include producers beginning to move away from using the crop as livestock feed, and distilleries no longer using rye malt on a large scale (Slinkard and Knott, 1995). In more recent years, however, the crop has seen another resurgence in popularity in Canada, with total rye production reported as being over 470 thousand tons, which is a height of production not having been seen since the 1990's. (FAO Stat, 2022). One possible explanation for this resurgence may be due to an increase in popularity for health foods, as foodstuffs like rye bread have several health benefits not seen in more common wheat-based products (Bondia-Pons *et al*., 2009). The rye grain is mainly used for human consumption, livestock feed, and the malting industry. Recently, rye is also grown as a fodder crop, as raw material for the bioenergy industry, or used as green manure (Newell and Butler, 2013; Galán *et al.*, 2020).

2.1.2. Impact of grain physical characteristics on yield

The yield of a cereal harvest is largely determined by the physical characteristics of the grain. These parameters include the physical dimensions of the grain, including grain width and length, and weight. Although kernel number is often the most important factor for grain yield (Fischer, 2008), grain weight is also an important characteristic as it relates to the amount of photosynthate products accumulated into a single kernel. A higher grain weight increases crop quality, with large and well filled grain contributing towards a superior end product than poorly filled grain.

The quantity and quality of the yield is not the only important trait impacted by the physical parameters of rye grain. It can also play a role in the survival rate and stress tolerances of the rye plant as well. In many crops, larger seed sizes correspond to an increase in overall survival rates, with the plants germinating from these larger seeds having higher tolerances to stress and result in mean higher productivity for the producers (Manonmani and Sundaram., 2014; Kawade *et al*., 1987). In part this is due to a larger seed having access to more energy for the initial period of germination and growth, allowing the

plant to become established more quickly and thus, be overall more vigorous. This in turn would lead to the plant becoming more resilient to stress causing factors. For wheat, it has been shown that the agricultural plots that were sown with wheat grain of a large size had significantly higher yield, in both biomass production and grain production, than those that had been sown with grain that was notably smaller in size (Zareian *et al.*, 2013; Shahwani *et al.*, 2014).

Grain production in cereals is impacted by more than just the tolerance of the plant to abiotic stress factors. Research has found that a reduction in plant height for winter wheat relates to an increase in overall yield, without negatively impacting the plant's low temperature tolerance (Limin and Fowler, 2000; Bahrani *et al.*, 2021). While a similar significant relationship between height reduction and yield increase has yet to be identified in winter rye, the current data available suggests that it would not have a negative impact upon the crop's cold tolerance.

2.2. Usages of rye products

2.2.1. Human consumption and health benefits of rye

The use of rye for human consumption is comparable to that of other domesticated cereal crops. The seed of the plant, the rye grain, is the main yield of the crop, with the remainder of the plant body potentially being utilized as straw for livestock, biofuel or green manure (Newell and Butler, 2013). Rye grain can be utilized in consumable products including bread, which is the most common human use of this cereal. In comparison to breads made from other types of cereals such as wheat, rye sourced bread is denser, and has a greater proportion of dietary fiber. Rye foodstuffs are most heavily consumed in northern Europe, where in some areas, it can account for 40% of dietary fiber (Bondia-Pons *et al*., 2009).

Rye contains a number of phenolic acids, which are hydroxylated derivatives of benzoic and cinnamic acids, which each have their own potential contribution towards improved human health, including serving as antioxidants, anti-inflammatory, and anti-

carcinogenic compounds (Bondia-Pons *et-al*., 2009). One of the beneficial phenolic compounds found in rye are lignans, which are classified as phytoestrogens due to having a similar molecular structure to estradiol (Bondia-Pons *et-al*., 2009). Alkylresorcinols are part of a group of 1,3-dihydroxy-benzene derivatives, that among the currently cultivated human consumed cereals, are only present in high levels in wheat and rye meal (Barron et al, 2007; Ross et al., 2003). These compounds have been noted to have some anti-oxidant properties (Bondia-Pons *et-al*., 2009).

Along with foodstuffs, rye grain can also be used in the production of alcoholic beverages, such as whiskey and beer. The distillation of rye grain into these products is a process similar to that used for other grains such as wheat. The grain is malted, a process which sees the grain partially germinated, then dehydrated and milled in order to alter the enzymatic content of the grain to a state more suited for fermentation. This process is effective for rye, as the grain is relatively easily damaged for future extraction during the malting process, but has a high-water uptake rate and a high potential extraction value. Once malting has concluded, the wort, a liquid extracted from the milled malt, is retrieved and fermented. The fermentation process can vary depending both on the techniques of the brewer enacting the process, and the desired end product, as while both beer and whiskey can be produced from rye grain, whiskey production is a more complex process (Wang *et al*., 2018). Rye whiskey is predominantly produced in the United States and Canada. It has been reported to have a distinct, spicy flavor in comparison to whiskey created from other grain types (Whiskeypedia, 2020). Globally, the whiskey market reached a net worth of over $80 billion US in 2022, with rye-based whiskey making up roughly 6% of the total market value (2023). The Canadian market for rye alcohols has seen steady growth recently, starting from 2017 (Statista, 2023).

2.2.2. Rye as animal/livestock feed

Aside from human-intended consumable products, rye grain may also be utilized as animal feed. A study by Knipfel (1969) found that rye possessed more biologically valuable

protein content for livestock when compared to wheat. Increased dietary protein from various sources has been found to allow for improved growth rates for livestock, such as pigs or poultry (Johannsen *et al*., 2023 and Tallentire *et al*., 2018). Rye grain would be an additional supplementary source of protein for some producers, particularly as more conventional high protein food stocks like soybean are not widely grown in some more northern regions of Europe (Van Krimpen *et al*., 2013). With Canadian livestock alone consuming over 28 million tons of feed stocks in 2021, this could be a means of meeting future demand (ANAC, 2023). A potential drawback however, is that rye grain contains a quantity of water soluble pentosans which inhibit the nutritional quality of the grain for some livestock. This issue is not insurmountable, as it can be alleviated with additional enzymatic supplements to counteract the pentosan content of the rye (Fengler, 1986). Additionally, drawbacks like these may be alleviated as a result of future breeding efforts, which could potentially see the economic value of this crop increase further.

2.2.3. Additional usages of rye

In addition to consumable products, winter rye may also be utilized as a cover crop in an agricultural system (Newell and Butler, 2013). Studies have found that integrating winter rye into a crop rotation system can improve and stabilize yields for many different crops including corn or maize (Rai *et al.*, 2023; Martinez-Feria et al, 2016). Rye cover crops have been recognized to provide benefits for the quality of the soil, including: enhancement of soil organic matter, reducing the rate of nutrient loss from leaching, and mitigating soil erosion (Blanco-Canqui *et al*., 2015). A rye cover crop may also benefit an agricultural system through suppressing undesirable weed growth. Due to the plant being partly grown before winter, then becoming dormant until spring, winter rye will grow relatively quickly in comparison to early spring weed species. The rye regrows from crown tissue once winter is over, while spring-seeded species need to grow from seed, giving the winter rye a head-start. This early establishment can act as an inhibitor towards the weed population in a field, which when combined with other mechanical and/or chemical techniques, may improve yields of a subsequent primary crop (Ateh and Doll, 1996).

There are a number of ways in which winter rye can provide support to an agricultural system. One of the more varied is through its impact upon different qualities of the soil it is grown in. The crop can be used for regulating the concentrations of soil nutrients such as nitrate or improving the water storage capacity of the soil in a field. For example, a winter rye cover crop can reduce the environmental impact of nitrates leaching into local water systems through trapping some of the excess nitrate in their tissues (Waring *et al*., 2020). It is further shown that consecutive use of winter rye as a cover crop, the soil water content and storage capacity of test plots is improved without negatively impacting the yield of the primary crop (Blanco-Canqui *et al.*, 2015; Basche et al 2016). While the dietary and agricultural support aspects of winter rye are important, another valuable aspect of this crop is its relatively high tolerance of environmental stressors, including low to freezing temperatures.

2.3. Rye low temperature tolerance strategies

2.3.1. Impact of freezing conditions on rye yield

One of the most important qualities of any cereal crop for a producer is the grain yield of the plant. Freezing conditions that may come during winter are a potential source of stress that could negatively impact regrowth in the spring and subsequently yield. The impact of low temperatures during anthesis or grain filling may have on the quality and quantity of the rye grain varies greatly depending on the degree of injury. Studies performed on the impact of low temperatures on winter wheat found that plants stressed by freezing temperatures during heading suffered a variety of injuries that negatively impacted the yield. The injuries included damaged or dead tillers and stems, a reduction in the number of spikes produced per plant, and a reduced quantity of grain produced per spike (Zhu, 1986). Yield quantity may also be reduced through a reduction of overall grain weight, which may come about due to damage caused to the assimilative areas of the plant, like leaves and stems. If these organs are damaged or destroyed though freezing

stress some other stress causing factor, there will be few resources available for the plant to allocate to grain growth (Spiertz *et al*., 1971).

For cereals like wheat or rye, injury caused by freezing temperatures can stem from two primary factors, the intensity of the low temperature stress and the duration of that stress. The degree to which either stressor impacts the plant in relation to the other can vary depending upon the situation. One study found that out of two groups of cereals, the ones exposed to ambient temperatures 8°C colder accumulated cold related injury and deaths over ten times faster than the higher temperature group (Gusta *et al.*, 1997). These injuries have been observed to be slightly more likely to occur in the tillers of the cereal rather than the main body. It was also observed that the primary sources of yield loss for cereals like wheat exposed to freezing temperatures were the reduction in grain producing spikes being grown from the plant, and an overall decrease in the grain weight (Zheng *et al*., 2018).

In the specific case of rye, the issue of low temperature stress caused injury is similar to that of other cereals. Damage to the main body and tillers of the plant from freezing conditions can result in both a reduction in grain weight and number of spikes produced by a rye plant. These injuries can be mitigated to an extent through metabolic means, such as the synthesis of apo-plastic proteins in rye tissue suffering from cold induced stress to act as an inhibitor of ice crystal formation (Marentes *et al*., 1993; Griffith and Mcintyre, 1993).

2.3.2. Cold acclimation metabolism

The introduction of low temperatures, and the subsequent cold acclimation process can lead to a number of metabolic effects in a crop. Carbohydrates are the most abundant compounds present in rye grain, as they are an important part of the crops yield. They also play a role in the plant itself, being a primary product of photosynthesis. During the cold acclimation process, the processes regarding the metabolism and transport of sugars undergo significant alterations (Nägele and Heyer, 2013). These changes can include the

increased production of sucrose in the plant and converting this sucrose into a variety of different sugars such as glucose, raffinose, and fructose. These sugars aid in cold acclimation in multiple ways, including preventing cell membrane damage from ice formation, increasing membrane stability, and preserving the photosynthesis mechanisms in the plant (Schneider and Keller, 2009).

In addition to heightened concentrations of sugars, cold acclimation can lead to increased concentrations of multiple different metabolic compounds. Increased concentrations of the amino acid proline have been observed in cereals that have undergone the cold acclimation process (Kocsy *et al*., 2011). Proline plays several roles in a plant's response to stress, including acting as an osmolyte, an antioxidant, and a signaling molecule (Hayat *et al.*, 2012). For rye specifically, proline helps to prevent damage to the cytomembrane of the tissues of the plant during periods of cold and frost induced stress (Gong *et al*., 2020).

Another metabolic group associated with winter hardiness in cereals are flavonoids. There is a wide range of different compounds present in this group, which serve different roles in a living plant. Some flavonoids act as a form of pigmentation in different tissues, providing a range of varying hues for the plant (Khlestkina, 2013). For cereals like rye, flavonoids serve as part of a plant's response and defense against various forms of biotic and abiotic stress. Some flavonoids are capable of effective absorption of harmful UV radiation, helping to prevent or reduce the stress caused from excessive light exposure (Gould, 2004). Others do so through bonding with free heavy metal ions, preventing them from causing injury to the plant's tissues through oxidative stress (Hale *et al.*, 2001). Acting as an antioxidant is a major component of how flavonoids function to protect a plant from stress induced injury, being more efficient in the role than other observed antioxidants like ascorbic acid (Bors *et al*., 1994). One category of flavonoid which serves in this capacity are anthocyanins.

2.4. Anthocyanins in rye

2.4.1. General anthocyanin properties

The most recognized role of anthocyanins in plant tissues is as a hydro-soluble pigment compound, often occurring in the form of O-glucosides (Williams and Grayer, 2004; Fujiwara *et al*., 2018). Anthocyanins have been observed to influence pigmentation of flowers, seeds, and fruits in a wide variety of species. This aids in attracting animals and insects to the plant, which may serve as potential pollinators, or help spread the plant's seeds (Gould, 2004). The color of pigmentation that results from these compounds tends to be mainly hues of red and purple. While cereals such as wheat and rye naturally possess a low concentration of anthocyanins, their presence has been noted to substantially influence the cold and frost tolerance of the plant, and certain cyanins are associated with WFS (Bahrani *et al.*, 2019).

2.4.2. Anthocyanins and cold tolerance

Anthocyanins have antioxidant properties, which allows them to reduce the formation of free radicals (molecular compounds lacking an electron) as well as removing reactive oxidative species (ROS) created due to stress causing factors (Pietta, 2000). ROS are a group of molecular compounds, usually occurring as a by-product of intracellular metabolic activity, which can range from short lived species such as superoxide, to more long-lived species such as hydrogen peroxide (Finkel and Holbrook, 2000). ROS compounds lack a sufficient quantity of electrons to remain stable, and thus will readily react with their surroundings, taking electrons from other non-radical molecules, which both disrupts the molecules in question and may also initiate a chain reaction through the generation of additional free radical compounds. This ongoing reaction can injure living tissue directly through the activity of free radicals, along with the generation of lipid peroxides, which if catalyzed can disrupt multiple cellular functions. The various means through which ROS can cause injury to living tissues is known as oxidative stress. This stress factor can be a cause of injury to a number of different cellular components with non-radical properties,

such as lipid membranes, proteins, and nucleic acids (Bartoli *et al*., 1999). The removal or reduction in concentration of these free radical species from the tissues of a living plant can prevent damages such as oxidative stress from occurring. (Gliwa *et al.*, 2011). Oxidative stress induced by excess ROS is also a cause of reduced mitochondrial biogenesis, a complex cellular process which serves to mitigate several forms of stress causing factors itself (Chevtzoff *et al*., 2010). The presence of antioxidant compounds such as anthocyanins can mitigate this issue through both reducing the concentrations of ROS in the cell as well as stimulating mitochondrial biogenesis activity in general (Gliwa *et al.,* 2011). Antioxidant compounds reduce ROS concentrations though donating electrons to free radical compounds, reducing the ability of these compounds to damage and disrupt cellular functions. Qualities like these not only allow antioxidant compounds to directly neutralize free radical elements present in living tissue, but additionally help to stimulate further repair and/or prevent damage to mitigation functions in the plant. This has been observed in other cereals placed under abiotic stress like wheat, with anthocyanin compounds playing a similar protective role (Olenichenko *et al*., 2006). The beneficial nature of antioxidant compounds such as anthocyanins contributes to their value, both to the health of the rye plant as well as to the health of the humans that consume the rye grain.

2.4.3. Anthocyanin health benefits

Flavonoids possess a number of valuable properties in relation to improving human health. Some sub-groups of flavonoids such as flavones have been observed to provide improvements towards insulin resistance, thus reducing the likelihood of type 2 diabetes occurring (Jennings *et al*., 2014). Flavones have also been noted to play a role as anti-inflammation agents. The ability to aid in mitigating inflammation related damages caused from factors such as lipopolysaccharides is another health-related property attributed to a number of different flavonoid compounds, including flavones, flavanols, and anthocyanins (Agah *et al*., 2017). One of the more prominent health-related aspects of anthocyanins, are their relation to antioxidant activity (Hock *et al*., 2017). They are efficient free-radical

scavengers, with multiple bases and structures present in the compound like the hydroxyalkene component contributing to its antioxidant properties (Vitale *et al*., 2016).

In a similar manner to how they function in plant tissue, antioxidant compounds in help to prevent or repair cellular damage in the human body caused though the activity of free radicals and ROS by neutralizing and/or reducing the concentrations of these substances. This helps to mitigate the increased generation of these harmful substances from heightened or compromised metabolic activity in the body, making antioxidant compounds an asset to improving human health. Most flavonoids have some antioxidant properties, with some of the more well-known examples including flavones and anthocyanins.

Another additional health benefit of anthocyanins would be the active suppression and reducing the likelihood of cancerous growths. Certain anthocyanin compounds including delphinidin and cyanidin have been noted as being a potential anti-angiogenic agent, preventing the formation of new blood vessels that may feed into cancerous tissue (Khoo *et al*., 2017; Matsunaga *et al*., 2010). This would potentially both serve to prevent further expansion of a tumor, along with starving the existing cancerous mass of oxygen and nutrients (Khoo *et al*., 2017; Matsunaga *et al*., 2010). Anthocyanins have been observed to have anti-cancer properties towards multiple forms of cancer (Chen *et al*., 2015). Studies have found that anthocyanin compounds were suppressing the metastases of breast cancer cells through disruption of specific protein kinase pathways. The study observed that in their breast cancer cell cultures the anthocyanin compounds used, which included cyanidins and peonidins, suppressed the activation of several kinases which played vital roles in the invasion and expansion of tumors, slowing or even halting their growth. These results were suggested to imply that the anti-cancer properties of anthocyanins may be of value even for advanced stage cancer patients (Chen *et al*., 2015).

The anti-diabetic properties of anthocyanin compounds have also been a subject of considerable interest in research related to human health. Anthocyanins have been found in multiple studies to have a positive effect in increasing the rate of insulin secretion. In these studies, it was observed that anthocyanin compounds including: pelargonidin-3-

galactoside, cyanidin-3-glucoside, and delphinidin-3-glucoside all were related to increasing the rate of production of insulin in mammalian metabolism (Christion, 1993; Bolleddula *et al*., 2005). Similar benefits have been found in the consumption of rye-based foodstuffs, with both anti-diabetic and anti-obesogenic effects being observed in human trials (Sandberg *et al*., 2016).

One final notable health benefit of anthocyanins is their effect in improving visual health. Anthocyanin compounds have been stated to be important nutraceuticals in regard to human visual health (Hock *et al*., 2017). Previous studies have found that anthocyanin rich foodstuffs such as bilberries help in improving general visual acuity along with maintaining the functionality of photoreceptor cells under stress. (Miyake *et al*., 2012; Thiraphatthanavong *et al*., 2014). They also improve visual health through their anti-oxidative capabilities, one example of such being mitigating oxidative stress of the ocular tissues (Mok, 2014). For rye sourced metabolites, the benefit to eyesight directly is limited, though studies have found evidence of rye foodstuffs helping to improve cognitive functions and neuronal integrity (Sandberg *et al*., 2018). While the health benefits of anthocyanins are a valuable aspect of the winter rye crop, their potential ability to help predict factors that may impact the viability of rye is also very important.

2.5. Mass spectrometry of anthocyanins

Mass spectrometry is a form of analysis used to determine the molecular characteristics of chemical compounds. This is accomplished through the transfer of the molecules of a compound of interest into a gaseous, ionized state, either with a heat source for a neutral molecule, or the local elimination of all CO_2 around the sample for pure amino acids and peptides (Laskin and Lifshitz, 2006). To employ an older though still valid definition, “The basic principle of mass spectrometry is to generate ions from either inorganic or organic compounds by any suitable method, to separate these ions by their mass-to-charge ratio (m/z) and to detect them qualitatively and quantitatively by their respective m/z and abundance” (DMGS, 2022). Once this is done, techniques such as

fluorescence spectroscopy are employed to determine the physical structure and dynamics of the compound.

Early research using mass spectrometry in the 1980's primarily relied upon laser spectrometric methods, which while yielding impressive results, were noted to interpret the different spectra results less clearly from a given sample, making the geometric information regarding the structure of the compound being studied by these methods potentially imprecise (Cable *et al*., 1987; 1988ab). Later developments in the field would see the advent of alternative techniques for mass spectrometry, including microwave based spectroscopic methodologies, and quadrupole-time of flight (QTOF) mass spectrometry (QTOF-MS) (Laskin and Lifshitz, 2006), which was the form of mass spectrometry used in this study.

As previously discussed, the basic concept of mass spectrometry is to convert a sample into a gaseous, ionized state, and then gather information about the molecular compounds in that sample using varying spectroscopic methodologies. QTOF is a type of mass spectrometry where the mass-to-charge ratio of the sample ions is measured whilst the ions are held in a stable orbit by an electric field generated by four parallel electrodes. These ions are all accelerated with a single known quantity of kinetic energy, towards a detector that is a known distance from the starting point of the ions (Royal Society of Chemistry, 2022). This results in generation of ions that possess a lower mass to accelerate faster, thus reaching the detector before any ions with greater mass. Other methods of mass spectrometry were not employed in this study both due to incompatibilities with the current samples and the previous success found by Bahrani *et al*. (2019) using this method. Some mass spectrometry methods such as gas chromatography have limited viability for non-volatile samples, while others like Laser Desorption are optimized for a different form of sample (Herod, 2010) than what were employed in this study. Using this specific method of mass spectrometry in this study would aid in gathering a precise understanding of the molecular mass of the compounds present in samples, allowing for the software used in the mass spectrometry analysis to make estimates of the specific molecular composition and structure.

The potential identification of specific molecular sequences of interest in the rye grain samples was one of the advantages of utilizing this method of analysis for the study. Previous research into rye anthocyanin composition had identified a number of molecular compositions for certain compounds of interest for estimating winter hardiness (Bahrani *et al.*, 2019). The methodologies employed would allow for the potential isolation and identification of both anthocyanin compounds previously identified by Bahrani *et al*., 2019, as well as other compounds that may occur in the samples used.

The usage of a specific wavelength for the total anthocyanin concentration analysis is another factor which was used to aid in reading the results of the mass spectrometry analysis. Knowing what frequency, the potential anthocyanin compounds were likely to appear on would allow for the main focus to be upon the readings most likely to provide that information. This in turn allowed the identification any spikes recorded over the course of the mass spectrometry procedure run for the sample in question and utilizing the Analysis QT software employed at the Saskatchewan Structural Sciences Center to run the procedure, estimate the molecular composition.

2.6. Value of winter rye for study of cold tolerance

Winter rye is a cereal crop possessing a high level of winter hardiness. Between the high WFS value of most rye lines, alongside the other high tolerances of the crop to abiotic stress factors such as drought and poor soil, rye is a valuable resource for studies into improving crop tolerance. Due to the outcrossing nature of rye, there is a relatively high genetic diversity among rye lines as compared to other cereals (Li *et al.*, 2011). The genetic diversity is displayed by a wide range of tolerance levels towards stress causing factors, which is of advantage in trait studies (Li *et al.*, 2011). Relationships between flavonoid compounds like anthocyanins and winter hardiness in specific rye tissues have been identified in previous research (Bahrani *et al.*, 2019). With this foundation established, determining if this relationship exists in rye grain, is a viable next step in gaining understanding of the influence of anthocyanins upon rye WFS and possibly also grain properties.

Chapter 3

Materials and Methods

3.1. Plant material

A panel of 96 rye (*Secale cereale* L.) accessions were previously characterized for low temperature tolerance and winter field survival using a combination of freeze tests and field trials (Bahrani *et al.*, 2019, 2021). Since rye is a cross pollinated crop, the accessions are not fully homozygous. Therefore, each accession in this thesis is called a culton, which is a term initially recommended by Hetterscheid and Brandenburg (1995a, b) for a systematic group of cultivated plants such as rye. The same rye panel had also been characterized by genotyping by sequencing (GBS) to identify genomic regions associated with winter field survival and plant developmental traits (Table 3.1) (Båga *et al*., 2022).

3.2. Seed production in the greenhouse

Ten rye grains of each culton were planted in a five cm wide and ten cm deep wells of planting pot trays, then placed in a Conviron phytotron growth unit (Controlled Environments Limited, Canada). The soil used, LG3 Propagation Mix (Sungro Horticulture, Agawam, MA, USA), was supplemented with 7.3 g L^{-1} slow-release fertilizer Type 100 NPK 14-14-14 (Arysta Lifescience America Inc., Burton, OH, USA) and 1.0 g L^{-1} Micromax micronutrients (ICL Specialty Fertilizers, Dublin, OH, USA). The phytotron growth unit was initially set to 20°C, with a humidity of 50%, and 16/8 light/dark cycle with light irradiance of 250 µE m^{-2} s^{-1}. The rye seedlings were watered once every one to two days, with the intent of keeping the soil moist, but not flooded. After two weeks, the conditions were changed to 4°C, 50% relative humidity, 8/16 light/dark cycle, and 120 µE m^{-2} s^{-1} light intensity. The rye plants were watered once every four to five days, the extended interval between watering times being implemented to take the altered environmental conditions into account. After six weeks acclimatization, the plants were transplanted into six-inch pots and transferred to a greenhouse chamber and grown there to maturity. Greenhouse

Table 3.1: Origin and Winter field survival of 96 rye cultons					
Winter survival class	Culton	Origin	Growth habit	WFS BLUE Score*	LT_{50} °C**
Very high	Leth Coulee Rye	Canada	Winter	92.5	-26.8
	Gauthier	Canada	Winter	90.1	-26.2
	AC Remington	Canada	Winter	86.2	-27.0
	AC Rifle	Canada	Winter	85.9	-27.0
	Musketeer	Canada	Winter	83.0	-27.8
	SM 38R	Canada	Winter	77.5	-24.0
	Prima	Canada	Winter	77.0	-27.5
	Saratovskaja 4	Russia	Winter	71.8	-26.8
	SM 4R	Canada	Winter	71.0	-26.8
	Pearl	Denmark	Winter	69.5	-26.6
	Kustro	Canada	Winter	68.8	-25.8
	Kharkivska 95	Ukraine	Winter	67.9	-24.8
	Kharkivska 98	Ukraine	Winter	66.9	-24.0
	Esprit	Germany	Winter	66.3	-22.8
	Ponsi	Sweden	Winter	66.0	-24.8
	Hazlet	Canada	Winter	65.5	-23.6
	Antelope	Canada	Winter	65.3	-26.2
	Emerald	USA	Winter	65.2	-22.0
	Anna	Finland	Winter	64.5	-22.0
High	R003-4	Canada	Winter	64.3	-24.0
	Voima	Finland	Winter	64.2	-23.8
	Dakota	Canada	Winter	64.1	-26.7
	Sc-73	Canada	Winter	64.0	-22.4

	Animo	Netherlands	Winter	63.6	-25.2
	Caribou	Canada	Winter	63.6	-23.8
	Puma	Canada	Winter	62.4	-26.0
	Othello	Sweden	Winter	62.2	-22.0
	Rymin	USA	Winter	61.9	-23.4
	Adams	USA	Winter	61.5	-22.8
	Sangaste	Estonia	Winter	60.3	-23.0
	Visa	Finland	Winter	59.9	-24.2
	Vitallo	Germany	Winter	59.6	-23.5
	Halo	Germany	Winter	59.5	-26.2
	Balbo	Italy	Facultative	59.4	-26.0
	Frontier	Canada	Winter	58.6	-24.4
	Enzi	Finland	Winter	58.4	-22.0
	Explorer	USA	Facultative	58.4	-23.4
	Motto	Poland	Winter	58.0	-23.6
	Dankowskie Selekcyjne	Poland	Winter	56.7	-23.8
Moderate	Galma	Belgium	Winter	56.6	-22.6
	Cougar	Canada	Winter	56.1	-24.0
	Dominant	Netherlands	Winter	55.8	-23.6
	Dankowskie Nowe	Poland	Winter	54.9	-24.6
	Danko	Canada	Winter	54.2	-24.8
	ACE-1	Canada	Perennial	54.0	-19.4
	Dankowskie Srebrne	Poland	Winter	53.9	-24.2
	Carolkurz	Germany	Winter	53.2	-23.8
	Horton	Canada	Winter	53.1	-24.0
	Kodiak	Canada	Winter	51.8	-25.0
	GC-100	Russia	Winter	51.6	-23.0
	Ameilo	Poland	Winter	49.2	-20.8

	Sellino	Germany	Winter	48.5	-21.8
	R538	UK	Perennial	48.1	-21.6
	Protector	Germany	Winter	47.8	-22.4
	Toivo	Finland	Winter	47.5	-23.8
	Culpan	Russia	Winter	47.0	-22.6
	Hardy white spring rye	Austria	Winter	46.9	-21.6
	Maton	USA	Facultative	46.2	-19.5
Low	Stoir	Ukraine	Winter	43.7	-22.2
	Vaschod	Belarus	Winter	43.7	-21.2
	R550	Czech Rep.	Perennial	43.6	-21.4
	Reimann Philipp	Germany	Perennial	42.4	-21.0
	Oklon	USA	Facultative	40.9	-19.6
	Carsten	Germany	Winter	39.5	-18.0
	R903	Unknown	Perennial	38.9	-22.0
	Harach	Canada	Spring	38.8	-21.4
	Danae	Germany	Winter	37.1	-21.2
	Clse 35	USA	Winter	36.8	-20.2
	Gator	USA	Facultative	36.0	-23.2
	Elbon	USA	Facultative	35.9	-17.0
	L-286-R	Germany	Winter	35.7	-16.4
	R904	Unknown	Perennial	35.4	-19.8
	Syn 20-L	Germany	Winter	35.3	-21.8
	SR4A-S5	Canada	Spring	33.2	-17.6
	Dakold	USA	Winter	31.1	-20.5
	Wheeler	USA	Winter	31.0	-20.8
	M. Karlic CT2	Russia	Winter	30.5	-19.5
Very low	Wintergrazer 70	USA	Facultative	25.2	-20.2
	Petkus Kurzstroh	Germany	Winter	24.1	-19.0

	Gazelle	Canada	Spring	23.6	-19.0
	Petkus	Germany	Winter	22.9	-21.2
	Prolific Spring	Canada	Spring	22.1	-19.2
	Wren Abruzzi	USA	Facultative	20.0	-18.0
	Extra Early Rye1	Mexico	Spring	19.7	-16.4
	Somro	Germany	Winter	16.0	-18.8
	R1210	South Africa	Perennial	15.7	-16.0
	Baltia	Russia	Winter	15.6	-16.8
	R797	Poland	Perennial	13.2	-16.0
	Fl-Synt	USA	Spring	12.9	-16.4
	Ottawa Select	Canada	Winter	12.9	-16.8
	Gulzow Kunz CT1	Germany	Winter	12.4	-16.2
	Rogo	Germany	Spring	12.4	-16.2
	Florida 401	USA	Spring	71.0	-15.8
	L-145-N	Germany	Winter	0	-17.0
	L-145-P	Germany	Winter	0	-16.5
	L-18-R	Germany	Winter	0	-16.5

*Winter Field Survival (WFS) determined as Best Linear Unbiased Estimates (BLUE) scores from five field trials (Bahrani *et al.,* 2021). **Determined from freezing tests (Bahrani *et al.,* 2021).

conditions were set to 20-23°C, 50% relative humidity, and artificial lighting creating a 17/7 light/dark cycle with a light irradiance of 1,500 µE m^{-2} s^{-1}. The rye plants were watered every other day.

As the rye seedlings began to germinate, their development was monitored and documented. This was done though photographs of the seedlings as they develop, as well as labeling leaves on the main stem upon emergence to indicate the growth stage of the plant. For the leaf labeling the method used was the application of numerical symbols on each of the leaves of the first rye shoot, using a black sharpie. Another method considered was labeling the leaves using colored tape labels. These colored labels were utilized to help determine the position of the flag leaf on each plant during harvest.

As ten grains were seeded per culton, this on average resulted in two groups of five plants per culton. Once the rye approached reproductive maturity, each group of five plants was covered with a pollen net made from polyester (Vogel *et al.*, 2014). This was done to prevent cross-pollination between the different cultons. The dates on which pollen was first observed on the rye heads, and when the rye kernels harden in the heads was also recorded.

3.3. Seed production in the field

To provide additional data and strengthen the final results, seeds produced in the greenhouse were seeded in a canola stubble field conditions at the University of Saskatchewan Experimental Kernen Farm, Saskatoon, Saskatchewan Canada (52 10’ N, 106 30’ W, 457 m altitude). One hundred individual rye grains per culton and row were seeded using a tractor-pulled seeder on September 9, 2021. Seeding was done in three randomly placed rows barring shortages of viable grain for that culton. The rye field was fertilized simultaneously with the sowing, using 11-52-0 NPK fertilizer mix (50 kg/ha). On September 30, 2021, the rye produced an average of 2-3 leaves per plant and the germination frequency per culton was determined. The plants were left to overwinter under snow cover, which would be captured by stubble present in the plot. In the early spring, the field was fertilized with 34-0-0 NPK (50 kg/ha). On May 24, 2022, a survey of the plot was done

to assess the survival rate of the 96 rye lines sown. This was done though visual observation of each row that had been seeded and estimating what percentage of the original sown area still contained living rye individuals. The survival rates were determined.

3.4. Plant phenotypic measurements

Phenotypic data collected during this growth period includes plant height, spike length, and awn length at harvest. This was done using manual measurements in cm, with the plant height being recorded using a meter-length measuring stick, and the grain head length and awn length being recorded with a conventional 15 cm ruler. The grain was then harvested and used to determine hundred grain weight, grain length and width, and grain coat coloration. The grain length and width were determined by measurements of the average of the combined length and width of 10 grains in quadruplicate from each line of rye. This method of measurement was employed to produce grain phenotypic characteristic data of a higher statistical accuracy for analysis. The grain phenotypic characteristics data was collected using measurements recorded with a handheld ruler, and a weight scale (Mettler Toledo, Columbus, Ohio).

Once the field grown rye reached maturity, pollen nets were placed upon a section of each row to prevent cross-contamination. The primary objective of these nets placements was to cover the maximum number of viable individuals without compromising required space for further development. On average, five individual rye plants would be covered by each net. Due to time constraints, along with losses over the winter, only two replications of each line could be provided with a pollen net before active pollen production began. The rye was harvested from August 22-24, 2022, with all plants that had been placed under the pollen nets being hand harvested to reduce the risk of errors and cross contamination of grain. Only seeds from netted plants, those that had not experienced cross-contamination, were used for the later analyses. Many of the lines that were harvested suffered from extensive ergot infection. This was likely due to an increase of moisture in the microclimate created by the pollen net, resulting in more favorable conditions for the spread of ergot spores. Phenotypic traits of the rye plants including plant

height, spike length, and awn length were recorded using the same methods employed for the greenhouse grown plants. The harvested heads of each rye set were stored in an individually labeled paper bag. The heads were then threshed using a small threshing machine (Wheat Head Thresher, Precision Machine Controls Inc., USA), after which the grain was stored in labeled paper envelopes for analysis. Out of the 96 lines of rye initially sown, only 88 produced enough uninfected grain for further analysis. The grain of each rye line was then assessed for grain morphological characteristics, composition, and specific anthocyanin concentrations.

3.5. Color analysis of grain

A color gradient analysis was also performed on both the greenhouse grown and field grown rye. This was done at the University of Saskatchewan Field lab, using a MiniScan XE Plus (Hunterlab, Reston, Virginia, USA) to take readings of intact rye grain. The machine was set to daylight color analysis, with a readout of L*, a*, and b* (Lightness, Red/Green, Blue/Yellow, respectively). Calibration was done using a color standard plate with known values. Each culton was characterized in triplicate with the greenhouse grown rye having three individual plants randomly selected via random number generator, and the field grown rye having each replicate of a line done separately. Due to observed variance in the values, each replicate selected to be scanned was itself done in triplicate (technical replicates), to get a more accurate average value. Once the data was acquired, it was formatted into an excel spreadsheet for statistical analysis.

3.6. Anthocyanin extraction and quantification

To determine the anthocyanin concentration present in the winter rye cultons, a two-part procedure was employed. The initial step was to use a spectrophotometric method to determine the total concentration of anthocyanins in extracts prepared from grain samples (Bahrani *et al.*, 2019; Royal Society of Chemistry, 2022). Thereafter, the extracts were analyzed by high-pressure liquid chromatography (HPLC) followed by quadrupole-time of

flight (QTOF) mass spectrometry (QTOF- MS) to identify anthocyanin species (Bahrani et al 2019).

3.6.1. Total anthocyanin concentration

Samples of rye grains were rapidly frozen in liquid nitrogen and ground to a fine meal in liquid nitrogen using a mortar and pestle. The meal was weighed in triplicates of 100 mg each and suspended in 1 mL of a MeOH:1 M HCl (85:15 v/v) solution as described (Bahrani *et al.*, 2019). The anthocyanin content of the samples was extracted for 4 h by shaking using an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany) set at 150 r, min^{-1}. Cell debris was removed by centrifugation at 20,000 g for 20 min and the supernatant was used to determine absorbance at A_{530nm} using a Beckman DU 8000 spectrophotometer (Beckman Coulter, Brea, California, USA.). The anthocyanin concentration in the grain extracts was calculated using a calibration curve, prepared using samples with known concentrations of cyanidin-3-O-glucoside and pelargonidin-3-O-glucoside (Figure 1). All readings were done in triplicates (technical replicates) and the total anthocyanin concentration (µg mL^{-1}) was determined for each sample.

3.6.2. Anthocyanin characterization in grain extracts

The prepared grain extracts were concentrated to 0.1 mL using a Savant SpeedVac™ Vacuum Concentrator (Thermo Fisher Scientific), filtered through a 0.45 µm, 4 mm Teflon syringe filters, and then separated by HPLC followed by peak identification by QTOF-MS. The sample analyses were done using a MDS Sciex QSTAR XL LC/MS/MS TOF instrument (Applied Biosystems, Foster City, CA, USA) located at the Structural Sciences Center, University of Saskatchewan. Three different concentrations of cyanindin-3-glucoside and pelargonidin-3-glucoside (5, 10 and 50 µg mL^{-1}) were used in as calibration standards in the analyses. The QTOF-MS system was calibrated with the Sex Pheromone Inhibitor iPD1 (BACHEM California Inc., Torrance, CA, USA) and cesium iodide (Sigma-Aldrich Chemistry, Milwaukee, WI, USA) using the parameters optimized for rye anthocyanins (Bahrani *et al*

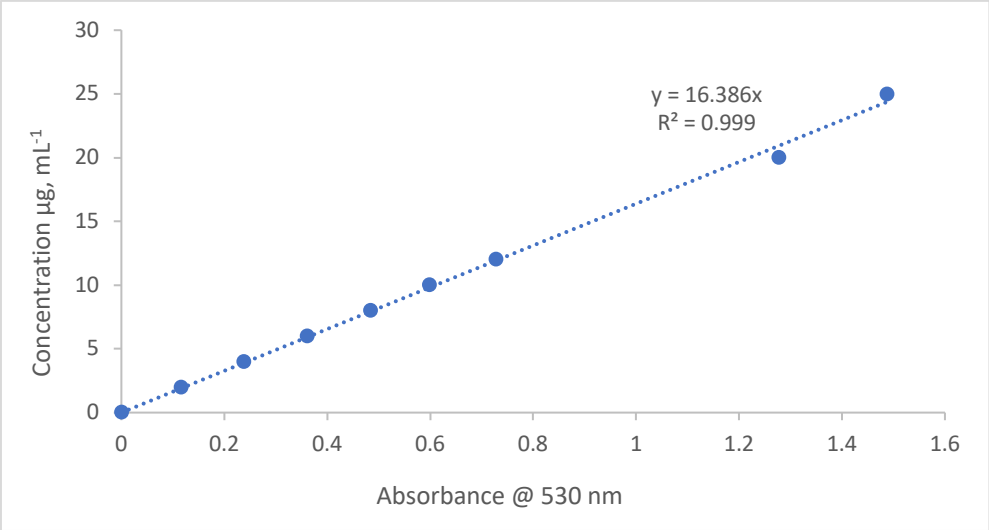


Figure 3.1: Standard curve for anthocyanin content using known concentrations of Cyanidin-3-Glucoside as standard.

2019). Samples of 10 µL were injected onto a 200 mm × 4.6 mm, 2.6 µm C18 Aeris column (Phenomenex, Torrance, CA, USA) using a flow rate of 0.25 ml/min and employment of a positive electrospray ionization mode. Elution was done along a gradient for 55 min using two mobile phases, phase A (1% formic acid in nanopure H_2O) and phase B (22.5% methanol, 22.5% acetonitrile in nanopure H_2O). The mass to charge ratio data generated from the QTOF/MS analyses was used to help identify individual anthocyanin compounds from an on-site library at the Saskatchewan Structural Sciences Center and Pubchem from the National Center for Biotechnology Information (https://pubchem.ncbi.nlm.nih.gov/).

HPLC grade methanol, hydrogen chloride, acetonitrile and formic acid used in the analysis were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HPLC water (0.22 µm; 18.2 mohm-cm) was prepared from distilled water using a Barnstead Nanopure system (Fisher Scientific, Waltham, MA, USA). The cyanidin-3-O-glucoside (Cya-3-Glc), cyanidin-3-O-rutinoside (Cya-3-Rut) and pelargonidin 3-O-glucoside (Pel-3-Glc) compounds diluted to 0–40 ng μL^{-1} concentration in methanol, 1 M HCl solution (85:15 v/v) were used as external standards.

As shown in a previous study of anthocyanins in rye grain extracts, certain anthocyanin compounds can be confirmed by the specific fragmentation patterns generated during an HPLC-MS analysis (Zykin *et al.*, 2018). In this study, the fragmentation pattern was determined for a select number of samples to confirm the presence of certain anthocyanin compounds in the lines run. The samples used in this procedure were evenly selected from all five winter hardiness categories of the rye panel, with a bias towards lines that gave strong readings in the previous mass-spec analysis.

3.7. Determination of starch content in grain

Rye grain was milled using an UDY cyclone mill (Udy Corporation, Fort Collins, CO, USA) equipped with a 0.5 mm sieve mesh to generate flour for grain starch determination. For each flour sample, a 100 ± 2 mg aliquot was added to a 50 mL tube and mixed with 0.2 ml 80% ethanol before 3 ml thermostable alpha-amylase solution (3ml; 300 U; company)

was added. The samples were then digested for 6 min in a boiling water bath while kept suspended by quick vortexing every two minutes. Thereafter, the 3.2 mL digest was supplemented with 4 mL 200 mM sodium acetate buffer pH 4.5 and 0.1 mL amyloglucosidase solution (20 U; company), which were mixed in by quick vortexing. Upon a 30 min incubation in a 50°C water bath, sample volumes were adjusted to 50 mL using 200 mM sodium acetate buffer pH 4.5. A homogeneous sample was generated by vortexing before quickly removing a 1 mL aliquot to a 2 mL tube containing diluted 1 mL of 200 mM sodium acetate buffer pH 4.5. Insoluble material was removed from the samples by centrifugation at 2,800 x g for 5 min and a 0.1 mL aliquot of the supernatant was mixed with 3 mL of GOPOD reagent (50 mL GOPOD stock solution reagent buffer in 1,000 mL Nanopure water). A reagent blank (0.1 mL sodium acetate) and a glucose control (0.1 mL glucose standard 1% w/v) were also prepared with the GOPOD reagent. All samples and controls were incubated at 50°C for 20 min and then measured for absorbance at 510 nm using a Beckman DU spectrophotometer (Beckman Coulter, Brea, California, USA). Once all data had been collected, the percentage starch on fresh weight basis, as well as dry weight basis in the rye grain were calculated per the following formula:

Percent starch (fresh weight) = sample absorbance X (100/glucose absorbance) X extracted volume "

Percent starch (dry weight) = starch percentage X (100/100-moisture percentage).

3.8. Determination of protein concentration in grain

For the protein concentration determination, ground rye grain prepared with liquid nitrogen was used in the same manner as done for the anthocyanin determination (section 3.6.1). Protein concentration in rye flour samples (250 mg) was determined by the combustion method with an FP-528 protein/nitrogen analyzer (LECO Corporation, St. Joseph, MI, U.S.A.). Percent protein (%P) concentration was obtained by nitrogen (%N) quantification with the following formula: %P = %N × C, where C is 5.7 for rye (AACCI Approved Method 46-30.01).

3.9. Data Analysis

The data was subjected to analysis utilizing R Studio version 1.2.1335 (RStudio team 2020) and Minitab software version 16 (Minitab, LLC, Pennsylvania, USA). The R Studio analysis was utilized for ANOVA analysis of the total anthocyanin concentration data along with the rye phenotypic characteristics data. These phenotypic characteristics included: grain length, grain width, hundred grain weight, rye plant height, rye head length, awn length, and stem height at harvest. The data was processed using a general linear model (GLM), with the intent of determining if there was any significant relationship between the total anthocyanin concentration of the rye grain and the rye phenotypic characteristics.

The Minitab software was utilized for statistic correlation analysis along with an analysis of variance for the data. Total anthocyanin concentration and rye phenotypic characteristics were the focus of the analysis, though WFS (Bahrani *et al*., 2021) were analyzed as well. The data for both green-house grown and field-grown rye was compiled into a single dataset, with an additional quantifying variable "Environment" added to differentiate between data from the same line of rye but grown under different conditions. The results of this correlation analysis would indicate which of the accounted variables had a statistically significant relationship with each other, and how strong these significant relationships were.

CHAPTER 4 Results

4.1. Rye grain production

4.1.1. Greenhouse environment

The seedings of 96 rye cultons established in the phytotron followed by growth in the greenhouse environment yielded mature plants from 53% of all seeds planted. Although the plant yield was low, it varied among the lines. For example, cultons like Vista and Vitallo established mature plants from all ten seeds planted, whereas Carolkurz and R903 produced only three and five mature plants, respectively, from ten seeds. Minor plant losses occurred during growth in the greenhouse, but most of the shortfall was caused by low germination and plant death during the cold acclimation period in the phytotron. The low plant establishment for some cultons were likely due to poorly filled and/or fungal infected seed used for planting. However, it was noted that cultons with high winter survival in the field were more likely to produce cold acclimated plants in the phytotron ($p > 0.05$). Cultons with very high WFS rye had an average survival rate of 74.7% and very low WFS rye showed an average survival rate of 69.4% upon cold acclimation.

During the vegetative growth phase in the greenhouse, incidents of aphid infestations were noted. However, these pathogen attacks did not become particularly severe as a soil-applied pesticide (Systemic Intercept 60pp, Terralink, Airdrie, AB, Canada) was applied to successfully control and prevent aphid spread. Overall, there were no visible outbreaks of disease-related symptoms among the various rye cultons in the greenhouse and more than 75% of the cold-acclimated rye plants matured satisfactorily and provided seeds of adequate quality for further study.

4.1.2. Field environment

The grain produced from 88 rye cultons grown in the greenhouse environment was used for seeding in the field. The germination frequency was found to be overall low (35%) and much lower than the germination frequency in the phytotron (77%). Although 64% of the

cultons had germination frequencies below 50%, some lines showed expected germination frequencies around 80-90% (Figure 4.1). From the data on 100-seed weight determined for the greenhouse produced grain, it was found that a higher seed weight related to a higher germination success in the field (R^2 = 0.205; Figure 4.1). Of the plantlets that successfully became established in the autumn, the survival rate in the spring varied widely. An 80-90% survival rate per culton was seen on the high end of the spectrum and a 3-6% survival rate on the low end (Figure 4.2). A comparison of the WFS frequencies determined in this trial with the average WFS determined in five previous trials (Bahrani et al. 2021) showed that the overall WFS in the current trial was about 13% lower than the five-year average. (Figure 4.3). Some cultons had a higher WFS in the current trial than in the previous ones, including SM 4R, Amilo, and R797, but several had lower WFS than in previous trials (Bahrani et al 2021).

The majority of the re-established plants in the spring reached maturity; thus, the yield of mature plants per culton depended on the initial germination frequency in the autumn and the plants' ability to survive frost during winter. While 26% of the sown rye cultons had a winter survival rate of less than 10% (Table 4.1), enough plants were established in the spring to provide at least one replicate of harvested grain for analysis. There were some additional losses of mature grain due to extensive ergot infections present on several netted plants, which seemed to be disproportionally afflicted as compared to the un-covered rye individuals from the same culton. The ergot-infected spikes yielded a small quantity of grain, or grain that was clearly afflicted by the fungal infection. During the threshing and grinding phases though, visibly unaffected grain was selected for analysis for all but eight of the rye cultons. Thus, grain from 88 field-grown rye cultons were available for analysis.

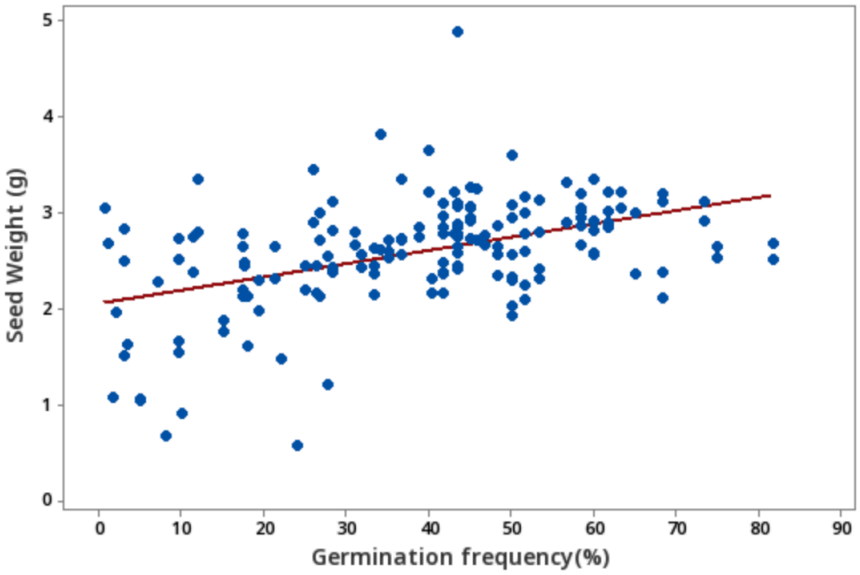


Figure 4.1 The influence of seed weight on the germination frequency of rye. Data from field seeding of 88 rye cultons in the autumn of 2021.

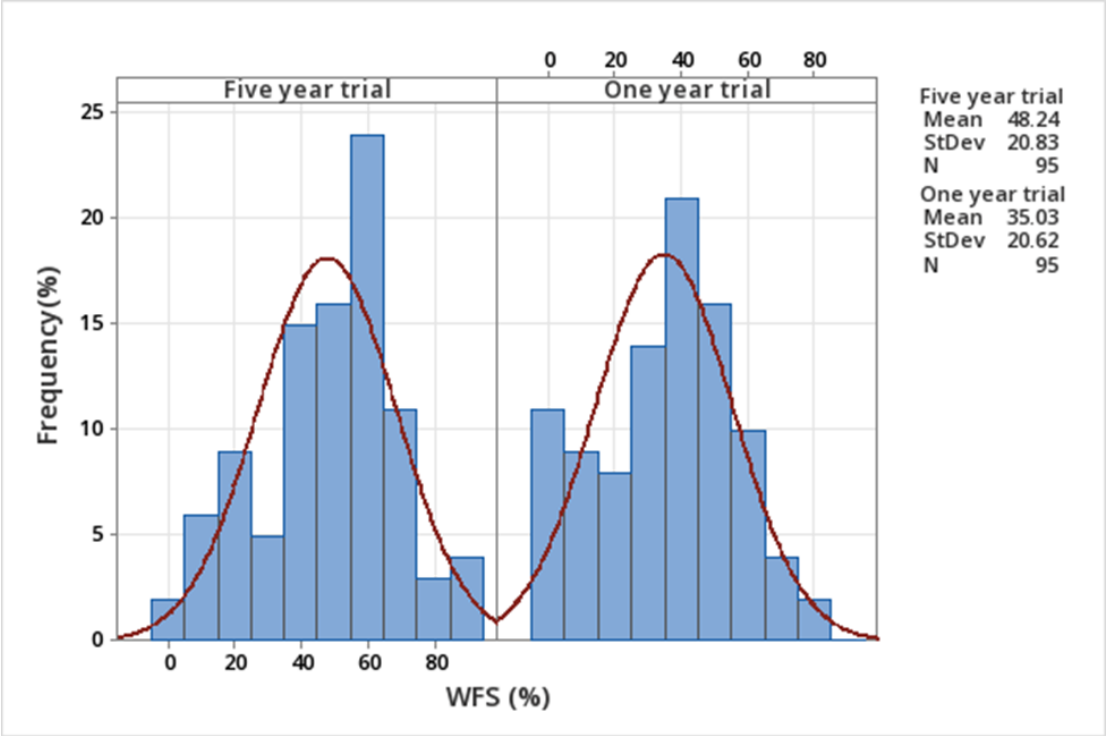


Figure 4.2: Histogram comparing the WFS of the five-year trial (Bahrani *et al.*, 2021) to the one-year trial undertaken in this study.

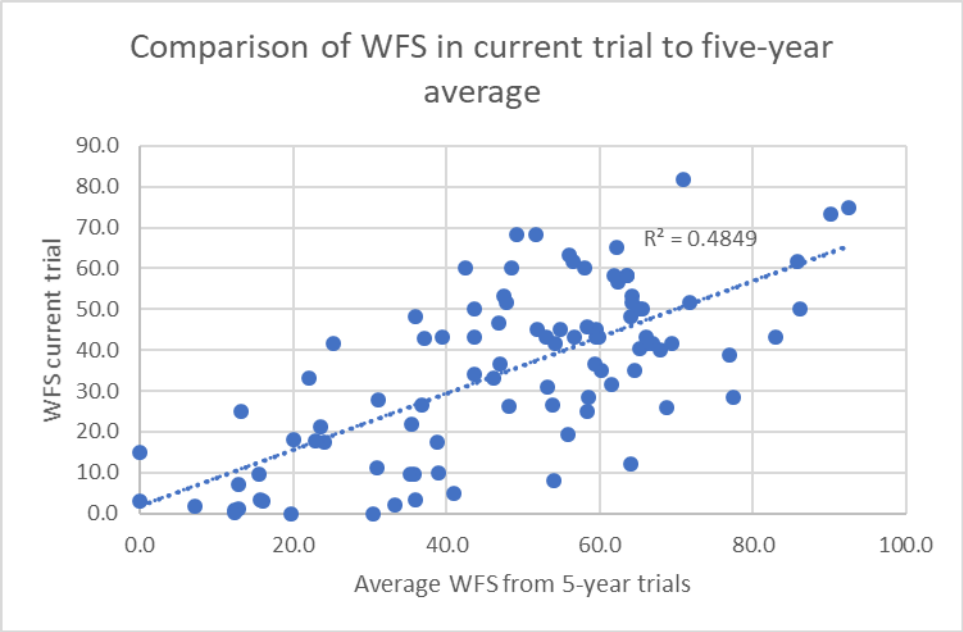


Figure 4.3 Comparison of WFS scores for rye cultons in current trial (2021–2022) to five- year average (Bahrani *et al.,* 2021).

Table 4.1 Winter field survival for 96 rye cultons studied in different trials.

Winter hardiness class	Culton	WFS	
		Five trials 2014-2019*	Current trial 2021-2022
Very high	Leth Coulee Rye	92.5	75.0
	Gauthier	90.1	73.3
	AC Remington	86.2	50.0
	AC Rifle	85.9	61.7
	Musketeer	83.0	43.3
	Sm 38R	77.5	28.3
	Prima	77.0	38.7
	Saratovskaja 4	71.8	51.7
	SM 4R	71.0	81.7
	Pearl	69.5	41.7
	Kustro	68.8	26.0
	Kharkivska 95	67.9	40.0
	Kharkivska 98	66.9	41.7
	Esprit	66.3	41.7
	Ponsi	66.0	43.3
	Hazlet	65.5	50.0
	Antelope	65.3	40.3
	Emerald	65.2	50.0
	Anna	64.5	35.0
High	R003-4	64.3	53.3
	Voima	64.2	51.7
	Dakota	64.1	48.3
	Sc-73	64.0	12.0
	Animo	63.6	58.3
	Caribou	63.6	58.3
	Puma	62.4	56.7
	Othello	62.2	65.0
	Rymin	61.9	58.3
	Adams	61.5	31.7
	Sangaste	60.3	35.0
	Visa	59.9	43.3
	Vitallo	59.6	45.0
	Halo	59.5	43.3
	Balbo	59.4	36.7
	Frontier	58.6	28.3
	Enzi	58.4	45.7
	Explorer	58.4	25.0
	Motto	58.0	60.0
	Dankowskie Selekc	56.7	43.3

Moderate	Galma	56.6	61.7
	Cougar	56.1	63.3
	Dominant	55.8	19.3
	Dankowskie Nowe	54.9	45.0
	Danko	54.2	41.7
	ACE-1	54.0	8.0
	Dankowskie Srebrne	53.9	26.7
	Carolkurz	53.2	31.0
	Horton	53.1	43.3
	Kodiak	51.8	45.0
	GC-100	51.6	68.3
	Amilo	49.2	68.3
	Sellino	48.5	60.0
	R538	48.1	26.3
	Protector	47.8	51.7
	Toivo	47.5	53.3
	Culpan	47.0	36.7
	Hardy white spring Rye	46.9	46.7
	Maton	46.2	33.3
Low	Stoir	43.7	43.3
	Vaschod	43.7	34.0
	R550	43.6	50.0
	Reimann Philipp	42.4	60.0
	Oklon	40.9	5.0
	Carsten	39.5	43.3
	R903	38.9	10.0
	Harach	38.8	17.3
	Danae	37.1	43.0
	Clse 35	36.8	26.7
	Gator	36.0	48.3
	Elbon	35.9	3.3
	L-286-R	35.7	9.7
	R904	35.4	22.0
	Syn 20-L	35.3	9.7
	SR4A-S5	33.2	2.0
	Dakold	31.1	27.7
	Wheeler	31.0	11.3
	M.Karlic CT2	30.5	0
Very low	38 Wintergrazer 70	25.2	41.7
	315959 Petkus Kurzstroh	24.1	17.3
	Gazelle	23.6	21.3
	Petkus	22.9	17.7
	Prolfic Spring	22.1	33.3
	Wren Abruzzi	20.0	18.0
	Extra Early Rye1	19.7	0
	Somro	16.0	3.0

R1210	15.7	3.3
118 Baltia	15.6	9.7
R797	13.2	25.0
Fl-Synt	12.9	1.0
445979 Ottowa Select	12.9	7.0
Gulzow Kunz CT1	12.4	0.7
Rogo	12.4	0.3
Florida 401	7.1	1.7
L-145-N	0	3.0
L-145-P	0	15.0

*Winter Field Survival (WFS) determined as Best Linear Unbiased Estimates (BLUE) scores from five field trials (Bahrani *et al.,* 2021).

4.2. Comparison of rye characteristics with winter hardiness

The developmental traits analyzed for green house and field grown plants were tested for relationships with cold tolerance traits (Tables 4.2 and 4.3). The relationships were also displayed by two sets of boxplot graphs, where one set of boxplots displayed data from the greenhouse-grown rye and the other data from the field-grown rye. This allowed a visualisation of the developmental/seed trait differences between different winter hardiness groups and growth environments.

4.2.1. Plant height

The boxplots of the rye plant height displayed several interesting points of interest. To begin with, the greenhouse grown rye showed larger variation in plant height and was notably taller as compared to the field grown rye, likely due to environmental differences in soil moisture, light intensity and quality (Figure 4.4). A greater degree of variation in plant height was seen in cultons with lower winter hardiness for both greenhouse and field grown cultons (Figure. 4.4). Field-grown cultons with the lowest WFS were on average shorter than plants with higher WFS scores. In contrast, cultons with the lowest WFS were on average the tallest among the greenhouse-grown plants. As WFS is known to be closely integrated with plant responses to light conditions (Novak et al. 2016), the contrasting heights in the two environments may have resulted from different light intensities and wavelengths. A positive correlation between plant height and WFS (R^2 0.34; $p < 0.001$) was noted for the rye population in a study by Bahrani et al (2021). In this one-year trial, a similar correlation was noted for the field grown cultons ($R^2 = 0.26$; $p < 0.001$, Table 4.3). However, plant height of greenhouse grown plants did not significantly correlate with WFS BLUE scores (Table 4.2). Overall, the developmental and grain related traits in field grown rye showed higher correlations to WFS than traits of greenhouse grown rye (Tables 4.2 and 4.3).

4.2.2. Spike and awn length

For the spike length at time of harvest, several observations were made. When comparing the greenhouse grown rye to the field rye, it was also observed that the spike length for greenhouse grown plants was greater across all winter hardiness levels (Figure 4.5a). The spikes of rye cultons with a low or very low winter hardiness level showed a tendency towards shorter spikes than those from higher winter hardiness categories. A correlation analysis showed the spike lengths of both greenhouse and field grown plants were significantly ($p < 0.001$) and positively correlated with plant height, awn length, and seed weight, respectively (Tables 4.2 and 4.3). Spike lengths of greenhouse plants were also positively correlated ($p < 0.001$) with seed width and WFS ($R^2 = 0.386$; $p < 0.001$; Table 4.2). Notably, no significant correlation was noted between spike length and WFS in the field grown plants ($R^2 = 0.057$; Table 4.2).

Awn lengths were overall longer on cultons grown in the greenhouse as compared to the field grown rye (Fig 4.5b). However, awn lengths did not display any clear trend among the different winter hardiness classes. The higher plant heights, spike lengths, and awn lengths for plants grown in the greenhouse as compared to the field could possibly be attributed to the different light and temperature conditions in the two growth environments.

4.2.3. Grain dimensions and weight

The measured length and width of the rye grain displayed both variation and uniformity over the different sets of data. Grain from field plants of the three highest WFS classes were slightly longer, wider, and heavier as compared to grain from the two lowest WFS classes (Figure 4.6). This is seen with the field-grown seeds from the three highest WFS classes having an average 100-grain weight value of 2.76 g, while the two lowest classes had an average weight value of 2.3 g (Fig 4.6c). Seeds for the three highest WFS classes produced in the greenhouse weighed on average 2.72g and 2.36 g for two lowest classes; however, the length and width values for greenhouse grain did not show any clear trends

Table 4.2: Pearson correlations coefficients between cold hardiness and developmental traits determined for rye cultons grown in the greenhouse.

	Plant height	Spike traits		Seed traits					WFS
		Length	Awn length	Length	Width	Weight	% starch	% protein	
Spike length	0.258***								
Awn length	0.299***	0.45***							
Seed length	0.181**	0.079	0.178**						
Seed width	0.210***	0.236***	0.334***	0.246***					
Seed weight	0.324***	0.31***	0.457***	0.371***	0.443***				
Starch (%)	0.091	0.042	-0.093	0.077	-0.055	0.117			
Protein (%)	0.024	-0.127	-0.066	-0.022	0.058	-0.124*	-0.56***		
WFS	-0.064	0.386***	0.186**	0.101	-0.009	0.162**	0.186**	-0.205***	

[1/]WFS as determined by BLUE scores calculated from five years of trials (Bahrani *et al*., 2021). P-values: ***(<0.001), **(<0.01), *(<0.05).

Table 4.3: Pearson correlations coefficients between cold hardiness and developmental traits determined for rye cultons grown in the field.

	Germination (%)	Plant height	Spike traits		Seed traits					WFS
			Length	Awn length	Length	Width	Weight	Starch (%)	Protein (%)	
Plant height	0.434***									
Spike length	0.135	0.384***								
Awn Length	0.107	0.124	0.269***							
Seed length	0.362***	0.344***	0.218**	0.228**						
Seed width	0.279***	0.245***	0.165*	0.121	0.47***					
Seed weight	0.453***	0.358***	0.265***	0.2**	0.65***	0.789***				
Starch (%)	-0.082	-0.123	-0.207**	-0.065	-0.181*	0.068	0.026			
Protein (%)	-0.381***	-0.03	0.013	0.01	-0.169*	-0.256***	-0.41***	-0.255***		
WFS	0.615***	0.255***	0.057	0.031	0.26***	0.279***	0.383***	-0.01	-0.309***	

[1/]WFS as determined by BLUE scores calculated from five years of trials (Bahrani *et al*., 2021). P-values: ***(<0.001), **(<0.01), *(<0.05).

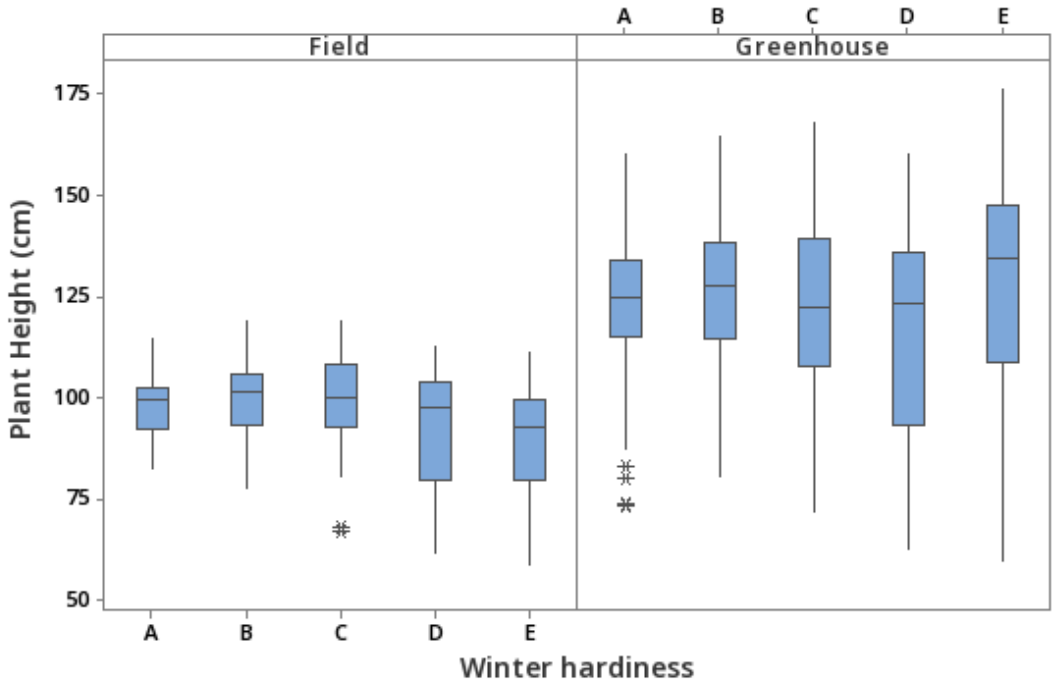


Figure 4.4: Relationship between plant height and WFS for rye cultons: Ranges of plant heights for 96 greenhouse (left panel) and 88 field (right panel) grown cultons, respectively, are shown. The winter hardiness classes are A=very high, B=high, C=moderate, D=low, and E=very low WFS. The plots show median (horizontal bar), interquartile ranges (boxes), ranges (whiskers), and outliers (dots) for WFS scores among the rye cultons.

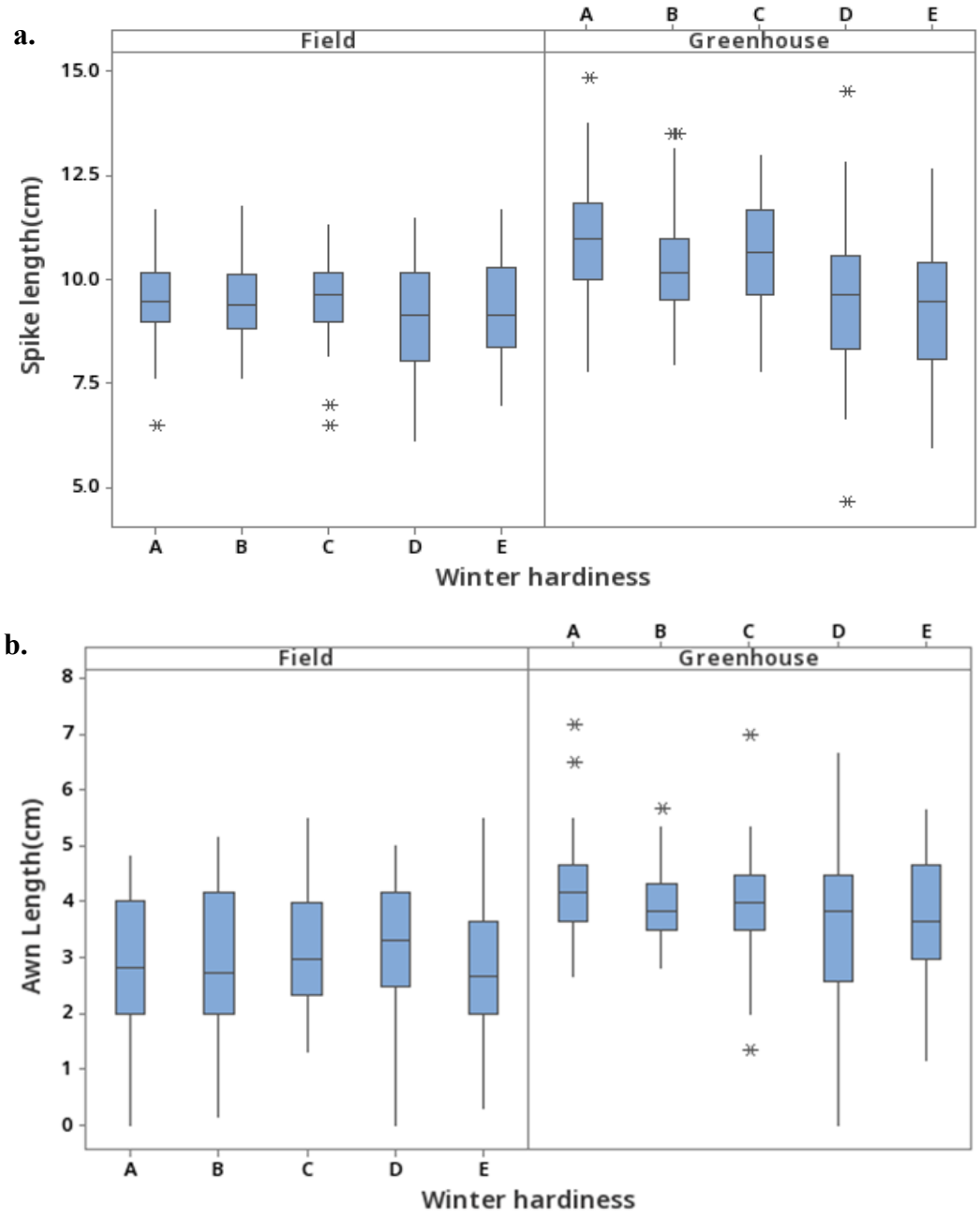
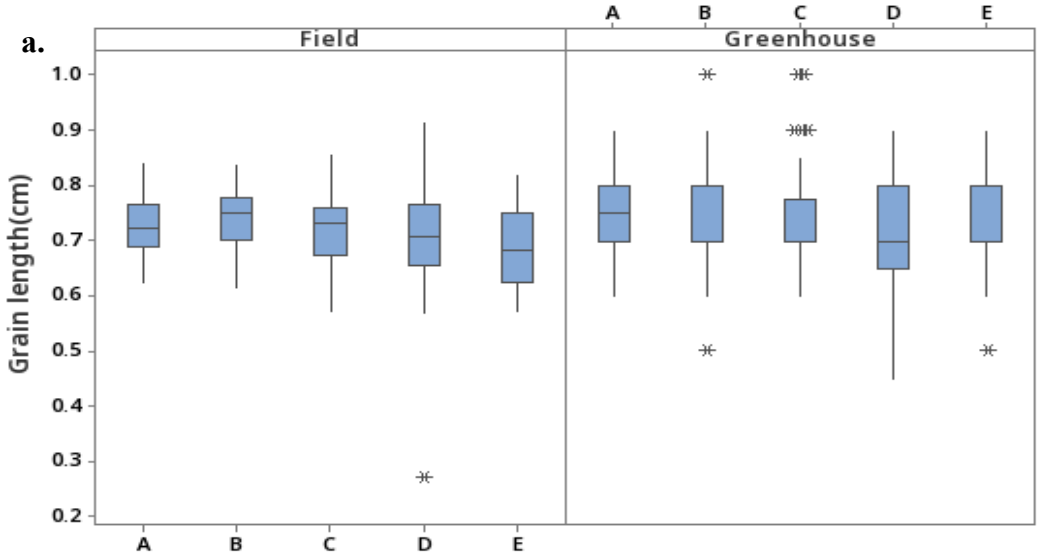
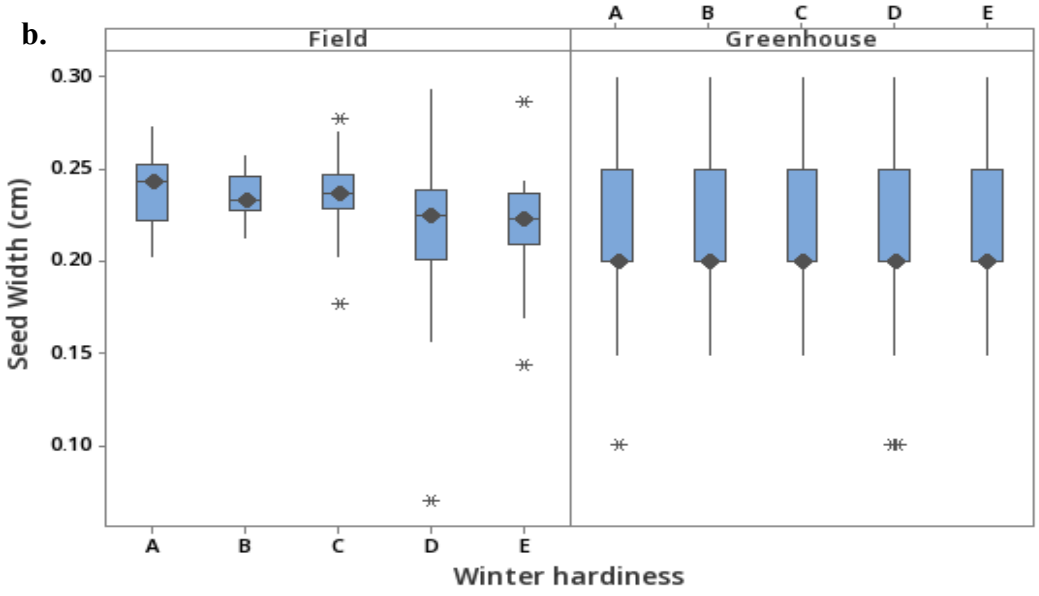


Figure 4.5: Box-plot analysis of spike and awn lengths among rye cultons of different WFS classes. The box and whisker plots show spike length (a) and awn length (b) measured for cultons grown in the field (left panel) and greenhouse (right panel). Median (horizontal bar), interquartile ranges (boxes), ranges (whiskers), and outliers (dots) for trait frequencies are shown. The winter hardiness classes are A=very high, B=high, C=moderate, D=low, and E=very low WFS.

a.


A B C D E
Field
Greenhouse
Grain length(cm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
A B C D E

b.


A B C D E
Field
Greenhouse
Seed Width (cm)
0.30
0.25
0.20
0.15
0.10
A B C D E
Winter hardiness

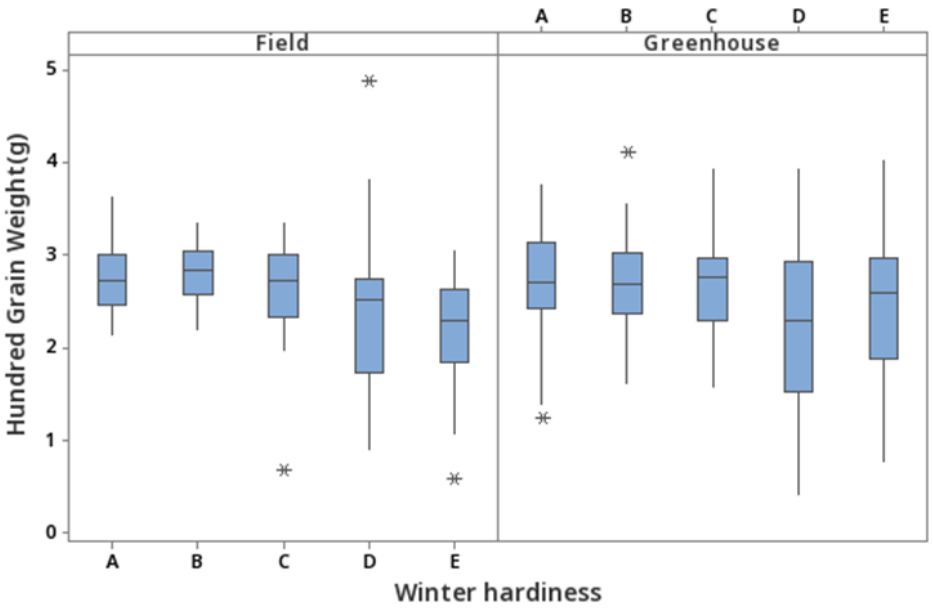


Figure 4.6 Relationship between grain morphology traits among different rye WFS classes. Plots display data for (a) grain weight, (b) grain width and (c) 100-seed weight obtained from field (left panel) and greenhouse (right panel) grown rye cultons. The WFS classes are very high (A), high (B), moderate (C), low (D), and very low (E). The plots show median (horizontal bar), interquartile ranges (boxes), ranges (whiskers), and outliers (dots) for trait frequencies among the rye cultons.

among the WFS classes, to the point where there was no visible variation on the graph plotted for width (Figure 4.6a and 4.6b). Thus, cultons with high winter hardiness seemed to have a more efficient grain filling during maturation both in the field and the greenhouse.

4.3. Grain starch and protein concentrations

The starch concentration in field-grown grain ranged from 30% to 71% with an average value of 57±0.43 %, whereas the greenhouse produced grain had starch concentrations from 42 to 64% with average value of 53±0.3 %. Starch is the major component of the rye grain and generally varies from 57 to 65% for rye (Hansen et al 2004). The higher starch concentration in field grain supports a more efficient grain filling as compared to the greenhouse environment (Figure 4.7a). Cultons of the lowest winter hardiness class showed the highest total starch concentrations in field-grown grain yet having some of the lowest starch content in greenhouse-grown grain. Thus, the growth conditions present for the greenhouse grown rye like reduced time available for vernalization, seem to have affected starch accumulation negatively and especially cultons with low WFS.

The protein percentage range for the greenhouse grown rye was from 12.6% to 26%, with an average value of 17.6±2.4% (Figure 4.7b). Field grown rye protein ranged from 10.7% to 23.2% with an average value of 15.5±1.9% and the values were more consistent than those of the greenhouse grown grain (Figure 4.7b). As generally found for cereals, the protein and starch concentrations were inversely correlated for both greenhouse (R^2=-0.56, p<0.001; Table 4.2) and field grain (R^2=-0.26; p<0.001; Table 4.3).

4.4. Grain color analyses

For the color analysis, the three categories of data were each compared with WFS individually. The L^* value which correlates with lightness / darkness varied around 50+/- 1.9 for most of the cultons, although grain from a few cultons had much lower or higher values, including

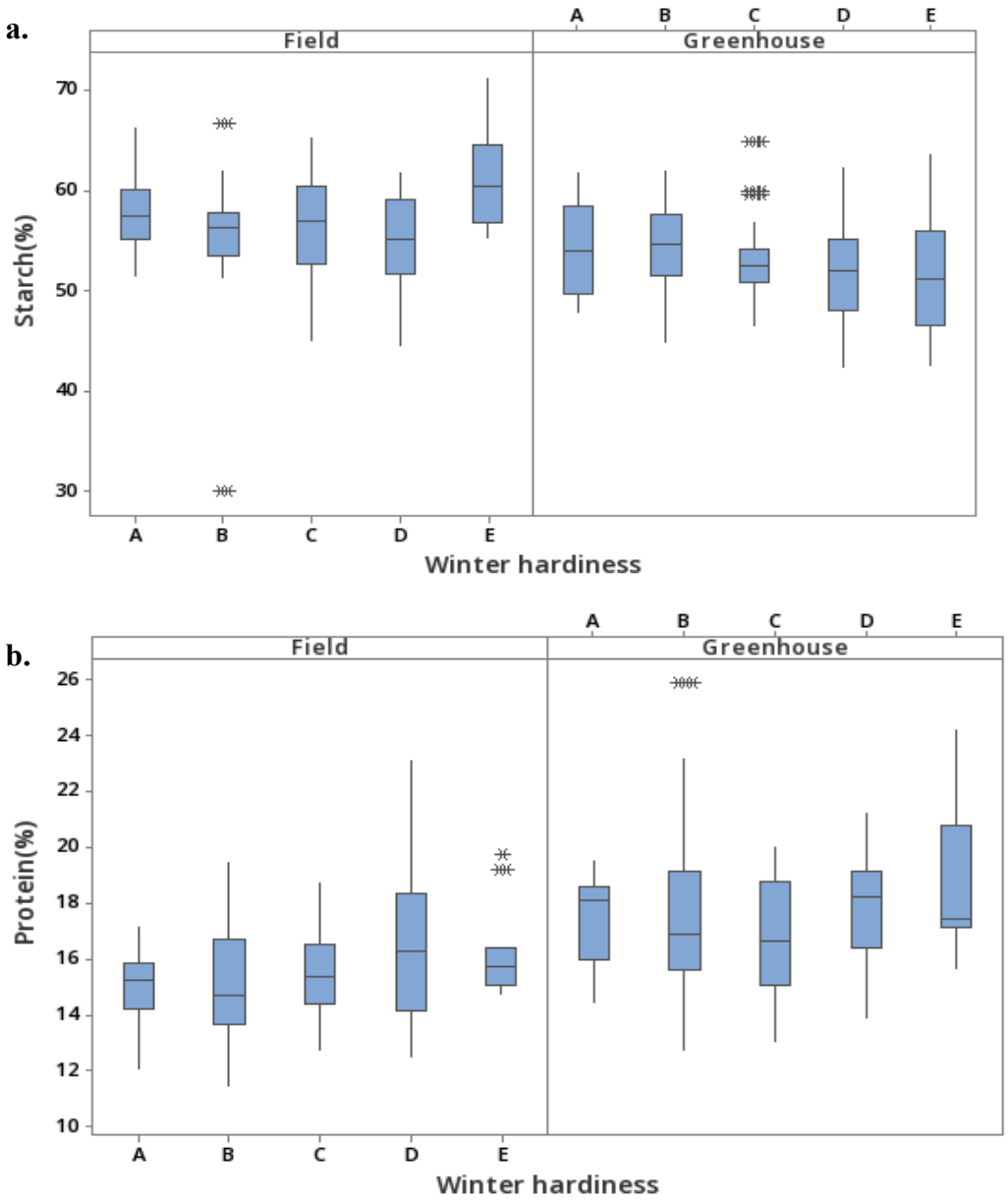
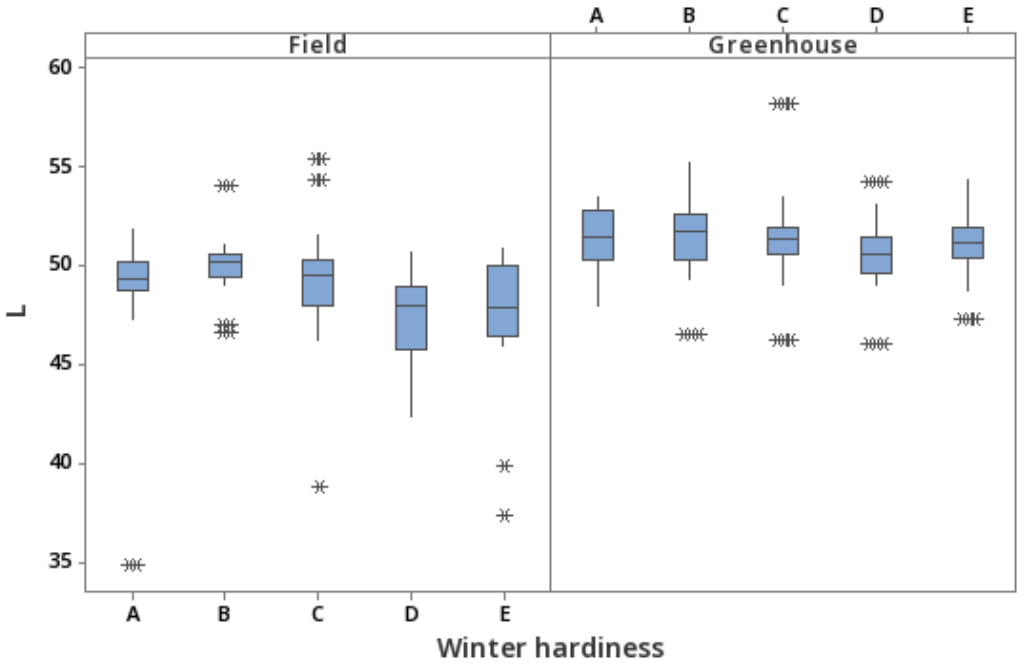
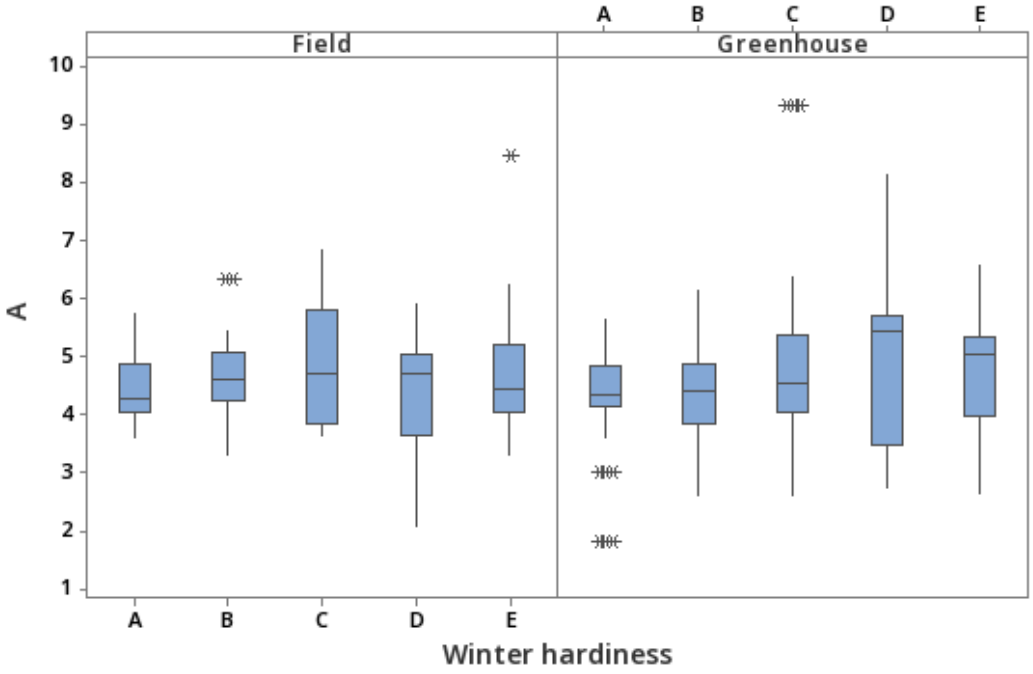


Figure 4.7: Relationship between major grain components and rye WFS classes. Box and whisker plots show (a) starch and (b) protein concentration in grain obtained from field (left panel) and greenhouse (right panel) grown rye cultons. The cultons are divided onto WFS classes with very high (A), high (B), moderate (C), low (D), and very low (E) winter hardiness. The plots show median (horizontal bar), interquartile ranges (boxes), ranges (whiskers), and outliers (dots) for trait data.


A B C D E
Field
Greenhouse
60
55
50
45
40
35
L
A B C D E
Winter hardiness


A B C D E
Field
Greenhouse
10
9
8
7
6
5
4
3
2
1
A
A B C D E
Winter hardiness

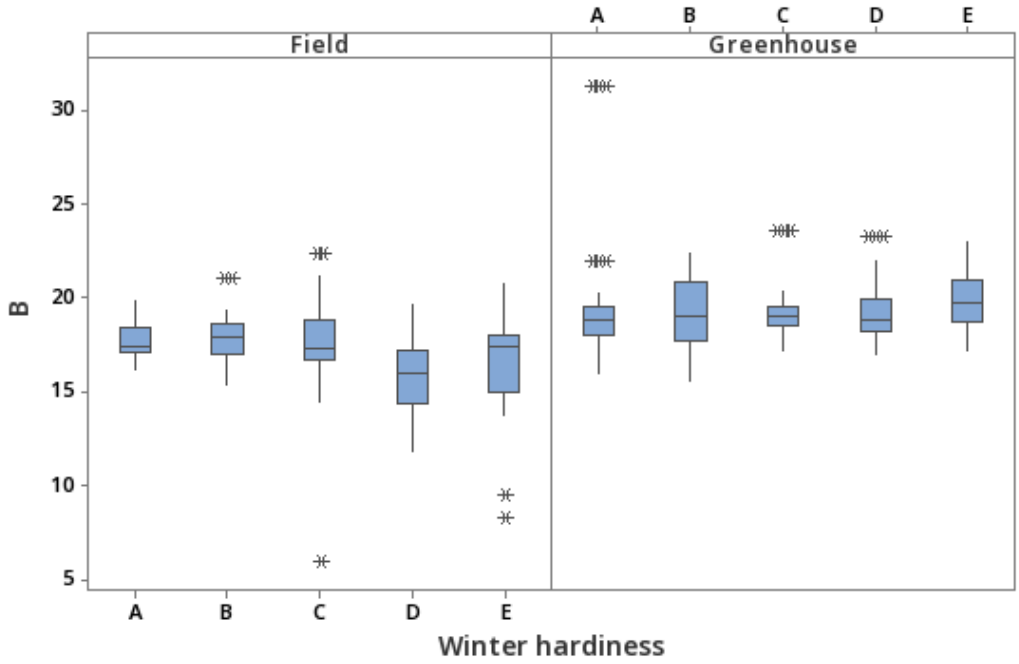


Figure 4.8: Relationship between grain color scores and WFS. Box and whisker plots show L (a), A (b), and B (c), color scores in grain grown in the field (left panels) and greenhouse (right panels). The cultons are divided onto WFS classes with very high (A), high (B), moderate (C), low (D), and very low (E) winter hardiness. The plots show median (horizontal bar), interquartile ranges (boxes), ranges (whiskers), and outliers (dots) for trait data.

R003-4 at 55.2, and Wheeler at 46.1. Grain from plants grown in the field, and especially the two lowest winter survival classes, showed slightly darker color than grain produced in the green house (Figure 4.8a). The a* value that is indicative of the green to red color, were all in the positive suggesting a tendency towards the red color (Figure 4.8b). Grain from the greenhouse grown plants of the two lowest winter hardiness classes had slightly higher a* values, indicating a higher tendency towards redness (Figure 4.8b). The b* values were all positive, which signified a higher tendency towards yellow than blue color. A stronger yellow color was noted for the greenhouse grain (Fig. 4.8c). Comparing the two environmental groups revealed field grown rye with low and very low winter hardiness having lower b^* color values than the other three winter hardiness levels. In contrast, greenhouse grown rye, the very low rye WFS cultons possess some of the highest b^* values among the different rye cultons of that group (Fig. 4.8c). In summary, the rye cultons in this study did not show much color variation therefore no correlations to WFS in the field or greenhouse produced rye seeds (Table 4.5 and 4.6).

4.5. Anthocyanin concentration in rye grain

The anthocyanin concentration and characterization were of particular interest for this study, given the importance of these two characteristics to the stated hypothesis. The greenhouse grown rye had a range of total anthocyanin concentration, that varied from 0.9 to 10.7 µg, mL^{-1}, with a mean value of 3.1±1.6 µg mL^{-1}. Field grown rye produced grain with total anthocyanin concentration ranging from 0.72 to 5.78 µg mL^{-1}, with a mean of 3.01±1.0 µg, mL^{-1}. Upon comparison of the greenhouse and field grown rye grains, some differences became readily apparent. The lowest concentrations of anthocyanins were observed in the very low WFS class grown in the greenhouse (Figure 4.9); however, greenhouse produced grain showed some of the greatest variation in anthocyanin concentration. For field grown rye, the very high WFS class had the highest concentrations of anthocyanins. In contrast, for greenhouse grown rye, the cultons with moderate winter hardiness tend to have the highest values, with very high and very low having some of the lowest concentrations of anthocyanins (Figure 4.9). The total anthocyanin

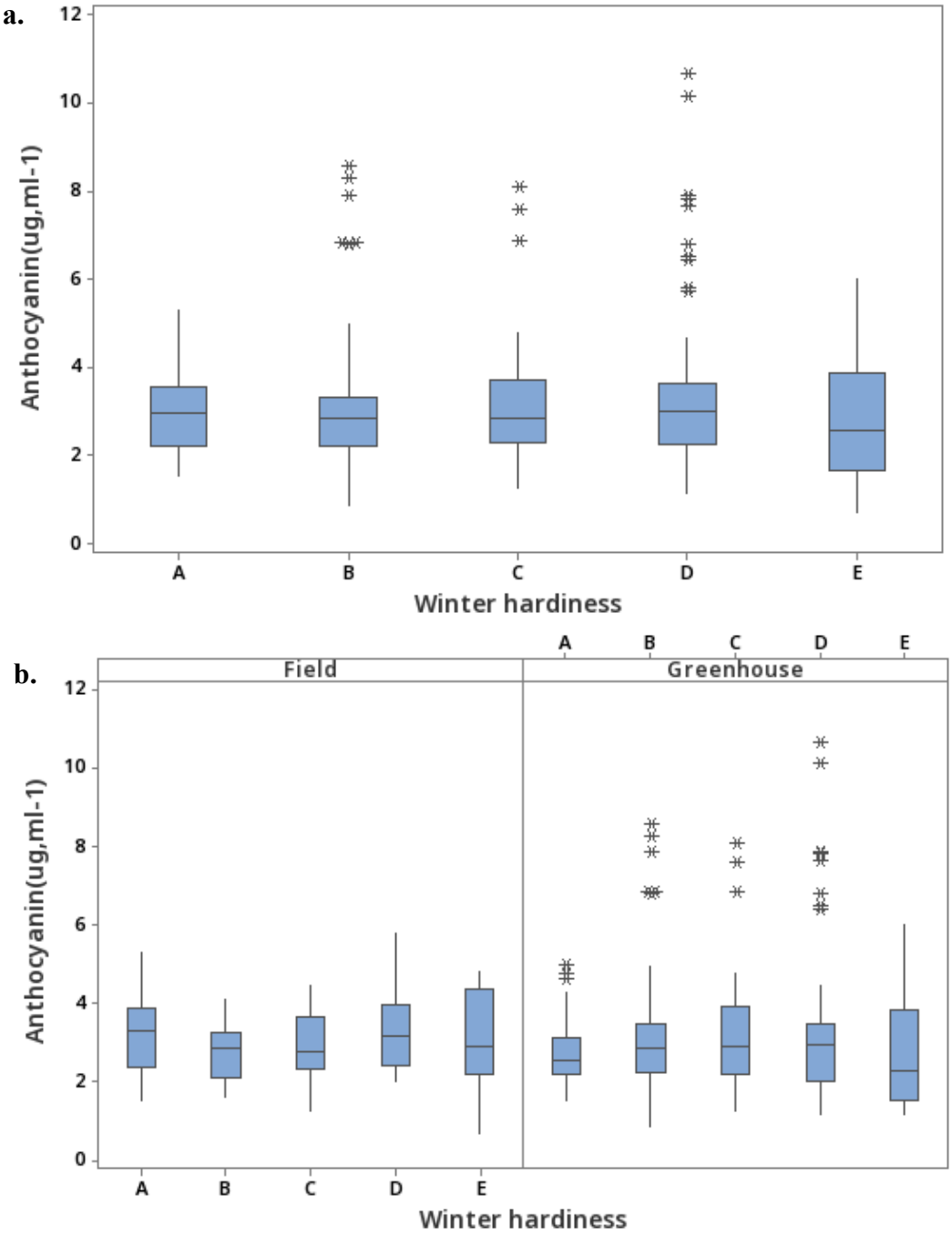


Figure 4.9: Relationship between grain anthocyanin concentration and WFS. The box and whisker plots show color scores in grain grown in the field (left panels) and greenhouse (right panels). The cultons are divided onto WFS classes with very high (A), high (B), moderate (C), low (D), and very low (E) winter hardiness. The plots show median (horizontal bar), interquartile ranges (boxes), ranges (whiskers), and outliers (dots) for trait data.

concentration was negatively correlated to the Hunter lab color parameters both in the field or greenhouse grown rye grains (Table 4.5 and 4.6).

4.6. Identification and characterization of anthocyanins

An HPLC-QTOF MS/MS instrument was used to identify and characterize anthocyanins and other components present in the grain extracts prepared from both greenhouse and field grown grain (Table 4.1). Three characterized anthocyanins, cyanidin-3-O-glucoside (Cya-3-Glc), pelargonidin 3-O-glucoside (Pel-3 Glc), and cyanidin-3-O-rutinoside (Cya-3-Rut), were used as external standards. The elution time for the standard peaks were noted and their identity confirmed by comparing their mass-to-charge-ratios (m/z) values with MS/MS spectral data available in the literature and allowing ±5 ppm difference between theoretical and experimental masses (Tables 4.9 and 4.10). Analyses of grain extracts by HPLC revealed between one to 13 peaks at $A_{530\,nm}$ for most of the rye cultons (Figure 4.10; Table 4.7). However, there were seven cultons that did not reveal any peak at A_{530nm} inferring either no or very low concentration of anthocyanins (Table 4.10). Besides the standard peaks, the chromatography peaks in the samples were identified as glycosylated forms of the three most common anthocyanidin aglycone structures: pelargonidin, cyanidin, and delphinidin. Several additional peaks at A_{530nm} were observed that suggested other anthocyanin compounds to be present in rye grains (Table 4.10). The majority of these unknown compounds however, were observed in only one or two rye cultons, indicating that there was not a common, easily observed relationship between them and other rye traits. The frequency of occurrence unknown compounds ranged from four separate observations of $C_{36}H_{35}O_9$, to seventeen separate observations like $C_{34}H_{32}O_5$, in the analysis of grain extracts (Table 4.11).

For further identification of the specific compounds observed, several different methods were employed. To begin with, PUBCHEM online database was accessed to determine if there were known compounds on record which directly matched the chemical formula deduced from the MS observations. This provided some level of identification for the results, though not a complete one. Some compounds observed in the rye samples could not be identified at all using this database, and very few known anthocyanin compounds were found (Table 4.11). The HPLC-

MS analysis performed found evidence of multiple anthocyanin compounds not detected by the conventional Mass-Spec analysis in the field rye lines tested. The results of this analysis found peaks corresponding to specific anthocyanin compounds described (Bahrani *et al*., 2019; Zykin *et al.,* 2018). In addition to these peaks, m/z readings from the samples which have been associated with specific anthocyanin fragments were also observed in the rye (Tables 4.9,4.10 and 4.11). Fragmentation ions associated with anthocyanin compounds such as delphinidin rutinoside and cyanidin rutinoside were observed in 16 of the tested rye cultons, though the specific compounds present varied between each culton. Cross similarity between identified anthocyanin compounds can provide an insight into sub-groups of anthocyanin compounds with their distribution pattern and probable winter hardiness.

It was observed that there were a greater number of anthocyanins in the field grown rye grain compared to the greenhouse grown rye grain. Four of the anthocyanins (cyanidin 3,5-O-diglucoside ($C_{27}H_{31}O_{16}$), pelargonidin 3-O-Glucoside ($C_{21}H_{21}O_{10}$), and pelargonidin 3-sambubioside ($C_{26}H_{29}O_{14}$), and Cyanidin 3-O–rutinoside ($C_{27}H_{31}O_{15}$), present in grains were also reported in rye living tissues (Table 4.9). Peonidin 3-sophoroside or peonidin 3,5-diglucoside, delphinodin rutinoside, delphinodin 3-0 glucoside, delphinidin 3-glucoside Dp-3-G) and two pelargonidin glucosides (pelargonidin3-O glucoside and pelargonidin 3-sambubioside) were present in grain but were not reported in rye crown or leaf tissues (Bahrani *et al*., 2019).

Other compounds identified included delphinidin 3-Glucoside, delphinidin 3-O-Glucoside, and peonidin rutinoside, which were also detected in the study by Zykin *et al*., (2018). There was also high prevalence of pelargonidin 3-O-Glucoside in the rye grain. This compound was also produced in cold-acclimated rye leaf tissue, but not correlate with high WFS (Bahrani et al 2019).

It was of interest to note that none of the characterized anthocyanins were detected in cultons that showed high anthocyanin concentrations (≥4 µg mL^{-1}), except Sm38R which showed the presence of peonidin-3-o-rutinoside and two delphinidin glucosides (Table 4.10). In the low winter hardiness group, Reimann Phillip, which showed very high anthocyanin concentration by the spectrophotometric method, showed six out of seven characterized anthocyanins (Table 4.10). The culton L-145-N which had very low winter hardiness, had high total anthocyanin

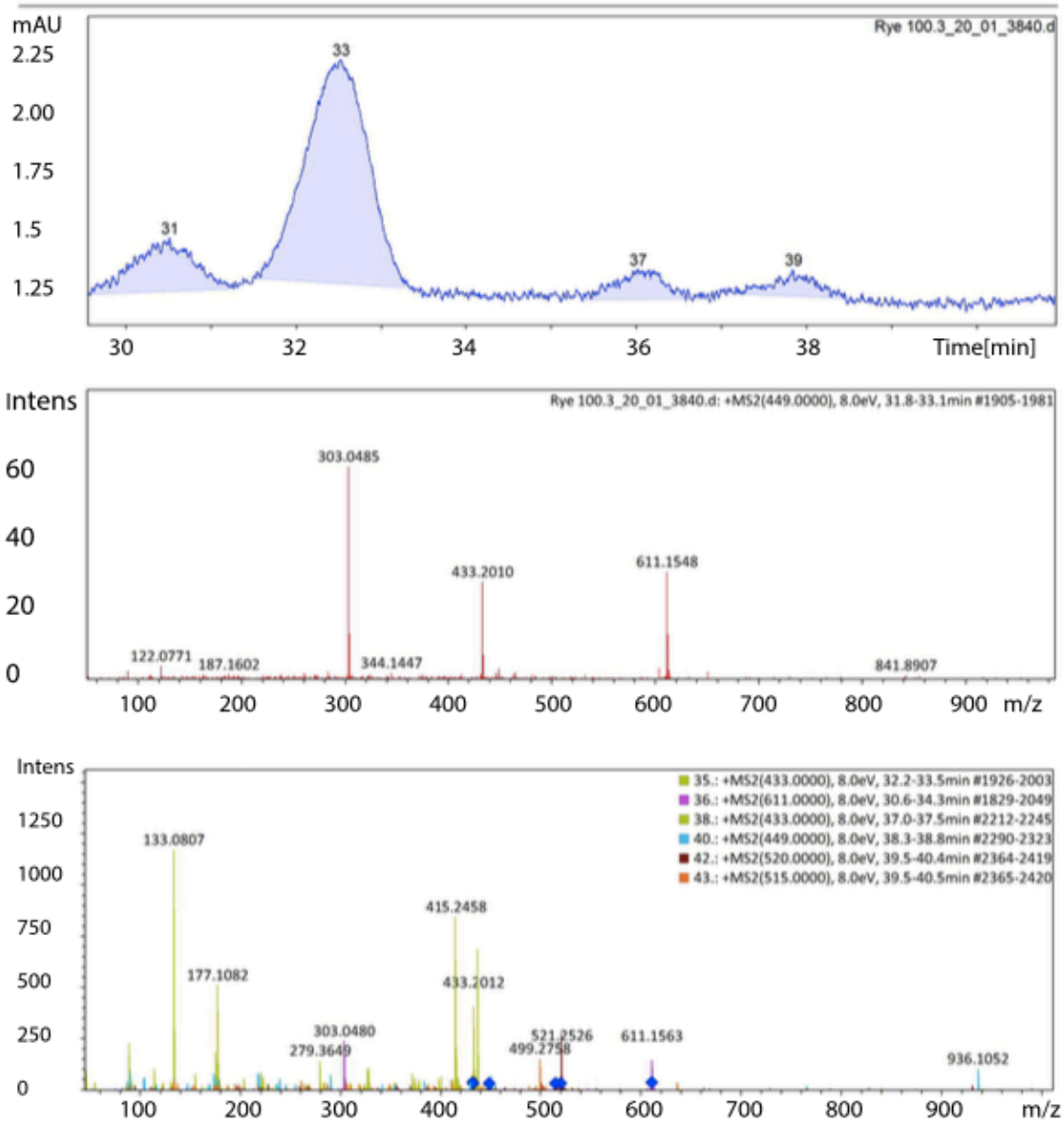


Figure 4.10: Analysis of grain extract by HPLC-QTOF MS/MS. Graphs display data obtained from analysis of grain extractable compounds obtained from culton Syn 20-L seeds produced in the field. Top graph displays the UV peaks (give wavelength) observed, the middle graph displays the m/z values of observed compounds at peak 33 of the top graph, and the bottom graph displays the overall m/z values for the entire sample, with specific wavelengths representing potential anthocyanins displayed in the top right of the bottom graph.

Table 4.4: Comparison of total anthocyanin content (μg, mL^{-1}) from greenhouse grown rye grain and field grown rye grain organized by winter hardiness level.

Winter Hardiness	Culton	Greenhouse Rye (μg mL^{-1})	Field Rye (μg mL^{-1})
Very High	Leth Coulee Rye	4.78	4.08
	Gauthier	2.24	1.68
	AC Remington	3.13	1.92
	AC Rifle	4.10	2.88
	Musketeer	3.67	5.08
	Sm 38R	2.56	2.91
	Prima	2.66	3.91
	Saratovskaja 4	2.36	3.20
	SM 4R	2.50	1.76
	Pearl	3.07	3.24
	Kustro	1.93	4.31
	Kharkivska 95	3.10	3.06
	Kharkivska 98	2.43	3.63
	Esprit	3.08	4.06
	Ponsi	1.57	1.58
	Hazlet	1.98	2.37
	Antelope	2.05	3.68
	Emerald	3.26	3.56
	Anna	2.20	3.70
High	R003-4	1.14	1.81
	Voima	2.28	2.52
	Dakota	6.82	2.09
	Sc-73	2.41	1.94
	Animo	4.46	3.33
	Caribou	3.32	3.11
	Puma	3.17	2.97
	Othello	3.07	1.88
	Rymin	1.97	2.24
	Adams	0.87	2.28
	Sangaste	3.54	3.33
	Visa	2.47	3.67
	Vitallo	3.07	2.86
	Halo	2.66	3.14
	Balbo	2.70	2.31

	Frontier	3.33	4.06
	Explorer	1.42	1.72
	Enzi	4.87	3.06
	Motto	2.27	2.92
	Dankowskie Selekc	8.24	3.97
Moderate	Galma	4.69	3.66
	Cougar	2.97	2.77
	Dominant	3.92	2.13
	Dankowskie Nowe	2.35	2.34
	Danko	2.45	3.32
	ACE-1	N/A	4.44
	Dankowskie Srebrne	2.88	2.32
	Carolkurz	7.51	4.44
	Horton	2.22	2.43
	Kodiak	2.15	1.47
	GC-100	3.54	2.62
	Amilo	4.54	2.77
	Sellino	3.79	3.15
	R538	2.95	3.65
	Protector	2.81	3.68
	Toivo	1.91	3.63
	Culpan	4.28	2.47
	Hardy white spring Rye	1.39	1.35
	Maton	1.83	4.10
Low	Stoir	3.14	4.44
	Vaschod	2.99	2.43
	R550	1.90	2.11
	Reimann Philipp	6.58	4.48
	Oklon	3.31	3.79
	Carsten	N/A	2.88
	R903	1.23	N/A
	Harach	3.65	3.24
	Danae	2.68	2.15
	Clse 35	2.54	2.36
	Gator	2.00	3.55
	Elbon	3.07	4.69
	L-286-R	10.41	3.30
	R904	1.85	N/A
	Syn 20-L	7.78	5.76

	SR4A-S5	3.17	2.45
	Dakold	1.82	2.34
	Wheeler	2.91	3.28
	M.Karlic CT2	4.37	N/A
Very Low	38 Wintergrazer 70	3.50	3.82
	315959 Petkus Kurzstroh	2.53	4.59
	Gazelle	4.94	2.61
	Petkus	1.78	2.74
	Prolfic Spring	1.25	2.09
	Wren Abruzzi	1.45	1.32
	Extra Early Rye1	3.64	
	Somro	3.17	3.20
	R1210	1.38	
	118 Baltia	1.34	2.92
	R797	N/A	N/A
	445979 Ottowa Select	1.79	3.22
	Fl-Synt	2.21	2.34
	Gulzow Kunz CT1	5.32	0.72
	Rogo	3.94	
	Florida 401	1.61	4.43
	L-145-N	5.27	4.70
	L-145-P	N/A	N/A

Table 4.5: Pearson Correlations of anthocyanin concentration and color score for greenhouse-grown rye cultons.

	Total anthocyanin	WFS BLUE score	L^*	a^*
WFS BLUE score	0.038			
L^*	-0.212***	0.127*		
a^*	-0.476***	-0.223***	-0.108	
b^*	-0.371***	-0.179**	0.278***	0.351***

Values stated here are R-values from correlation analysis. P-value: ***(<0.001), **(<0.01), *(<0.05)

Table 4.6: Pearson Correlations of anthocyanin concentration and color score for field-grown rye cultons.

	Total anthocyanin	WFS BLUE score	L^*	a^*
WFS BLUE score	-0.006			
L^*	-0.26***	0.161*		
a^*	-0.698***	-0.084	0.278***	
b^*	-0.508***	0.23**	0.754***	0.606***

P-value: ***(<0.001), **(<0.01), *(<0.05)

Table 4.7 Summary of number of anthocyanins observed in rye grain harvested from greenhouse and field, respectively, compared to that reported in crown and leaf tissue (Bahrani *et al.,* 2019).

Winter hardiness class	Culton	Growth habit	Number of anthocyanins identified		
			Grain (Greenhouse)	Grain (Field)	Leaf and/or crown tissue*
Very high	Leth Coulee Rye	Winter	1	10	4
	Gauthier	Winter	4	10	4
	AC Remington	Winter	3	5	0
	AC Rifle	Winter	3	5	2
	Musketeer	Winter	3	3	4
	Sm 38R	Winter	1	6	9
	Prima	Winter	2	6	6
	Saratovskaja 4	Winter	5	5	1
	SM 4R	Winter	2	5	5
	Pearl	Winter	2	4	2
	Kustro	Winter	1	5	3
	Kharkivska 95	Winter	1	4	10
	Kharkivska 98	Winter	1	5	4
	Esprit	Winter	2	5	0
	Ponsi	Winter	2	5	5
	Hazlet	Winter	2	6	6
	Antelope	Winter	3	3	9
	Emerald	Winter	3	5	0
	Anna	Winter	2	4	5
	R003-4	Winter	2	3	9
	Voima	Winter	2	6	2
	Dakota	Winter	2	4	9
	Sc-73	Winter	3	9	0
	Animo	Winter	3	6	6
	Caribou	Winter	-	3	5
	Puma	Winter	2	4	3
High	Othello	Winter	4	5	1
	Rymin	Winter	3	4	5
	Adams	Winter	1	2	4
	Sangaste	Winter	5	10	0
	Visa	Winter	3	5	4
	Vitallo	Winter	2	4	4
	Halo	Winter	2	5	10
	Balbo	Facultative	3	4	9

	Frontier	Winter	3	6	1
	Explorer	Facultative	3	6	0
	Enzi	Winter	4	3	0
	Motto	Winter	2	7	1
	Dankowskie Selekc	Winter	2	4	5
	Galma	Winter	2	6	3
	Cougar	Winter	3	5	4
	Dominant	Winter	1	4	4
	Dankowskie Nowe	Winter	1	5	1
	Danko	Winter	4	4	0
	ACE-1	Perennial	0	5	0
	Dankowskie Srebrne	Winter	2	4	8
	Carolkurz	Winter	3	4	1
	Horton	Winter	2	6	8
Moderate	Kodiak	Winter	2	4	3
	GC-100	Winter	4	4	3
	Amilo	Winter	2	3	3
	Sellino	Winter	2	4	2
	R538	Perennial	4	7	2
	Protector	Winter	4	4	1
	Toivo	Winter	2	4	4
	Culpan	Winter	6	5	2
	Hardy white spring Rye	Winter	1	8	0
	Maton	Facultative	3	6	5
	Stoir	Winter	4	6	1
	Vaschod	Winter	2	7	0
	R550	Perennial	2	10	1
	Reimann Philipp	Perennial	2	11	0
	Oklon	Facultative	2	4	3
	Carsten	Winter	-	4	10
	R903	Perennial	3	-	4
	Harach	Spring	2	8	4
	Danae	Winter	2	5	0
Low	Clse 35	Winter	3	7	2
	Gator	Facultative	1	4	0
	Elbon	Facultative	2	5	0
	L-286-R	Winter	5	11	6
	R904	Perennial	-	-	0
	Syn 20-L	Winter	2	8	4
	SR4A-S5	Spring	1	6	4
	Dakold	Winter	1	3	3
	Wheeler	Winter	2	13	0
	M.Karlic CT2	Winter	2	-	1

	38 Wintergrazer 70	Facultative	5	5	9
	315959 Petkus Kurzstroh	Winter	5	4	2
	Gazelle	Spring	5	5	4
	Petkus	Winter	2	4	4
	Prolfic Spring	Spring	2	6	2
	Wren Abruzzi	Facultative	1	4	4
	Extra Early Rye1	Spring	3	-	0
	Somro	Winter	4	6	3
Very Low	R1210	Perennial	6	-	0
	118 Baltia	Winter	3	5	10
	R797	Perennial	4	-	5
	445979 Ottowa Select	Winter	6	5	2
	Fl-Synt	Spring	5	4	0
	Gulzow Kunz CT1	Winter	2	3	6
	Rogo	Spring	2	-	2
	Florida 401	Spring	2	3	3
	L-145-N	Winter	-	8	2
	L-145-P	Winter	6	11	0

Table 4.8: Anthocyanins characterized in rye tissue (Bahrani *et al.,* 2019) and grains

Anthocyanin	Formula	m/z^+ experimental	m/z^+ calculated	Error ppm	RT (min)	Peak no
Cyanidin glucosides:						
Cyanidin 3-O-glucoside	$C_{21}H_{21}O_{11}$	449.1059	449.1078	4.2	24.41	6
Cyanidin 3,5-O-diglucoside	$C_{27}H_{31}O_{16}$	611.1622	611.1607	-2.5	25.47	7
Cyanidin *p*-coumaryl-diglucoside	$C_{36}H_{37}O_{18}$	757.1988	757.1974	-1.8	23.95	2
Cyanidin 3-O-rutinoside	$C_{27}H_{31}O_{15}$	595.1652	595.1657	0.8	27.88	15
Cyanidin 3-sambubioside	$C_{26}H_{29}O_{15}$	581.1525	581.1501	-4.1	25.93	9
Cyanidin 3-sambubioside-5-glucoside	$C_{32}H_{39}O_{20}$	743.2028	743.2029	0.1	23.12	1
Cyanidin 3-*p*-coumaryl-sambubioside	$C_{35}H_{34}O_{17}$	726.1772	726.1791	2.6	23.97	3
Cyanidin 3-xyloglucoside	$C_{26}H_{27}O_{15}$	579.1360	579.1344	-2.8	28.86	17
Cyanidin 3-O-acetylglucoside-5-O-glucoside	$C_{29}H_{33}O_{17}$	653.1738	653.1712	-4.0	30.23	18
Peonidin 3-O-rutinoside	$C_{28}H_{33}O_{15}$	609.1831	609.1814	-2.8	28.46	16
Peonidin 3-sophoroside or Peonidin 3,5 -diglucoside	$C_{28}H_{33}O_{16}$	625.1758	625.1768	1.696	33.30	3
Delphinidin glucosides:						
Delphinosidin rutinoside	$C_{28}H_{33}O_{14}$	611.16	611.16	N/A	17.8	1
Delphinodin 3-o Glucoside	$C_{21}H_{21}O_{12}$	465.1	465.1	N/A	20.3	2
Delphinidin 3-glucoside	$C_{21}H_{21}O_{12}$	465.102	465.103	-0.75	33.27	2
Delphinidin 3-O-*p*-coumaryl-monoglucoside	$C_{30}H_{27}O_{14}$	611.1408	611.1398	-1.6	25.62	8
Delphinidin *p*-coumaryl-diglucoside	$C_{36}H_{37}O_{19}$	773.1943	773.1924	-2.5	23.99	4
Delphinidin 3-O-caffeoyl-glucoside	$C_{30}H_{27}O_{15}$	627.1349	627.1344	-0.8	24.23	5
Malvidin 3-O-*p*-coumaryl-monoglucoside	$C_{32}H_{31}O_{14}$	639.1724	639.1708	-2.5	26.44	10
Petunidin 3-O-*p*-coumaryl-monoglucoside	$C_{31}H_{29}O_{14}$	625.1572	625.1552	-3.2	27.28	12
Pelargonidin glucosides:						
Pelargonidin 3-O-glucoside	$C_{21}H_{21}O_{10}$	433.1112	433.1129	3.9	27.52	14
Pelargonidin 3-sambubioside	$C_{26}H_{29}O_{14}$	565.1561	565.1552	-1.6	27.36	13
Pelargonidin 3-O-(6-O-malonyl-beta-D-glucoside)	$C_{24}H_{23}O_{13}$	519.1146	519.1133	-2.5	26.88	11

Table 4.9: Distribution of characterized anthocyanins in rye tissues (Bahrani *et al.,* 2019) and rye grains.

Anthocyanin	Abbreviation	Formula	Tissue		
			Leaf	Crown	Grain
Cyanidin glucosides:					
Cyanidin 3-O-glucoside	Cya-3-Glc	$C_{21}H_{21}O_{11}$	+	+	-
Cyanidin 3,5-O-diglucoside	Cya-3,5-diGlc	$C_{27}H_{31}O_{16}$	+	+	+
Cyanidin *p*-coumaryl-diglucoside	Cya-*p*-Cou-diGlc	$C_{36}H_{37}O_{18}$	+	-	-
Cyanidin 3-O-rutinoside	Cya-3-Rut	$C_{27}H_{31}O_{15}$	+	+	+
Cyanidin 3-sambubioside	Cya-3-Sam	$C_{26}H_{29}O_{15}$	+	-	-
Cyanidin 3-sambubioside-5-glucoside	Cya-3-Sam-5-Glc	$C_{32}H_{39}O_{20}$	+	-	-
Cyanidin 3-*p*-coumaryl-sambubioside	Cya-3-*pC*ou-Sam	$C_{35}H_{34}O_{17}$	+	-	-
Cyanidin 3-xyloglucoside	Cya-3-Xyl-Glc	$C_{26}H_{27}O_{15}$	+	-	-
Cyanidin 3-O-acetylglucoside-5-O-glucoside	Cya-3-AcGlc-5-Glc	$C_{29}H_{33}O_{17}$	+	-	-
Peonidin 3-O-rutinoside	Peo-3-Rut	$C_{28}H_{33}O_{15}$	+	+	+
Peonidin 3-sophoroside or Peonidin 3,5 -diglucoside	Peo-3-Sop or Peo-3-5-diGlc	$C_{28}H_{33}O_{16}$	-	-	+
Delphinidin glucosides:					
Delphinosidin rutinoside	Del-3-Rut	$C_{28}H_{33}O_{14}$	-	-	+
Delphinodin 3-o Glucoside	Del-3-O-Glc	$C_{21}H_{21}O_{12}$	-	-	+
Delphinidin 3-glucoside	Del-3-Glc	$C_{21}H_{21}O_{12}$	-	-	+
Delphinidin 3-O-*p*-coumaryl-monoglucoside	Del-3-*p*Cou-Glc	$C_{30}H_{27}O_{14}$	+	+	-
Delphinidin *p*-coumaryl-diglucoside	Del-3-*p*Cou-diGlc	$C_{36}H_{37}O_{19}$	+	-	-
Delphinidin 3-O-caffeoyl-glucoside	Del-3-Caf-Glc	$C_{30}H_{27}O_{15}$	+	-	-
Malvidin 3-O-*p*-coumaryl-monoglucoside	Mal-3-*pC*ou-Glc	$C_{32}H_{31}O_{14}$	+	+	-
Petunidin 3-O-*p*-coumaryl-monoglucoside	Pet-3-*p*Cou-Glc	$C_{31}H_{29}O_{14}$	+	-	-
Pelargonidin glucosides:					
Pelargonidin 3-O-glucoside	Pel-3-Glc	$C_{21}H_{21}O_{10}$	+	-	+
Pelargonidin 3-sambubioside	Pel-3-Sam	$C_{26}H_{29}O_{14}$	+	-	+
Pelargonidin 3-O-(6-O-malonyl-beta-D-glucoside)	Pel-3-6-Mal-βGlc	$C_{24}H_{23}O_{13}$	+	+	-

Table 4.10 Distribution of anthocyanins in grains of field grown and greenhouse grown rye plants.

Winter Hardiness Class	Culton	Cyanidins				Delphinidins			Pelargonidin		Uncharacterized compounds
		A	B	C	D	E	F	G	H	I	
Very High	Leth Coulee Rye										21,23,26,35,39,43,46,52
	Gauthier										4,6,7,36
	AC Remington			x		x			x		4,16,25,26,27,28
	AC Rifle										4,5,7,23,26,28
	Musketeer			x		x		x			11,24,25,26,30,41,46
	Sm 38R										4,5,12
	Prima	x									35,37,42
	Saratovskaja 4										2,3
	SM 4R										
	Pearl										1,11,27,37,40
	Kustro										17,22,41,45
	Kharkivska 95		x								9,13,34
	Kharkivska 98			x		x		x			10,18,20,35,42,45
	Esprit						x				28,30,45,52
	Ponsi										20,27,35,49
	Hazlet					x		x			11,14,16,34,43
	Antelope										4,7,13,27
	Emerald										4,8,26,30,35,43
	Anna										3,29
HIgh	R003-4										23,33,48
	Voima										1,10,29,34
	Dakota			x		x			x		2,14,34
	Sc-73	x									17,23,28,41,45,50,52,55
	Animo		x								12,14,15,23,25,27
	Caribou										27,28,30
	Puma										1,30,39,40,45
	Othello										10,20,24,27,34,45,47
	Rymin									x	20
	Adams										4,6
	Sangaste	x	x				x				37,39,49,52

	Visa							2,3
	Vitallo							2
	Halo							5,9,11,13,26,34,38
	Balbo							7,14,27
	Frontier							5,7,19,23,37,38,41
	Enzi							6,10,17,25,26,38
	Explorer							4,5,6,8,15,36
	Motto							17,23,30,31,32,35,38,43
	Dankowskie Selekc				x		x	x	10,31,36
Moderate	Galma								17,30,32,40,52,54
	Cougar								4,11,13,15,16
	Dominant			x	x			x	12,30,37,38,39
	Dankowskie Nowe				x			x	10,31,35
	Danko								6,9,12,23,27,32,41
	ACE-1								38,41
	Dankowskie Srebrne								7,16,33
	Carolkurz								7,16,31,32
	Horton								12,25,29,33,34,43,44
	Kodiak								17,18,21,26,27,40
	GC-100				x		x	x	5,7,15,19,24
	Amilo			x	x			x	4,6,23
	Sellino								1
	R538								
	Protector								17,40,51
	Toivo								
	Culpan								7,10,11,23,38,41
	Hardy white spring Rye								17,26,39,52
	Maton								18,22,25,32,35,38,46
Low	Stoir								2,3,4
	Vaschod	x				x			22,23,37,39,40,52
	R550								11,25,33,35,52,53
	Reimann Philipp	x	x	x	x	x	x	x	
	Oklon								23,24,25,26,27,35
	Carsten								7,33,34

	R903								
	Harach								9,24,25,26,27,41
	Danae								12,17,27,32,34,53
	Clse 35								6,7,10,25,35,37,51,53
	Gator			x		x		x	4,28,30,41,42
	Elbon								4,26,27,30
	L-286-R								
	R904								
	Syn 20-L	x			x	x	x	x	25,41,44,45,47,54
	SR4A-S5		x						18,20,24,44,45,47,48, 52,54
	Dakold								33,35
	Wheeler	x	x				x		42,47,49
	M.Karlic CT2								16,20,21,23,26,35,43,46
Very Low	38 Wintergrazer 70								3,5,6,7,23,41,51,52
	315959 Petkus Kurzstroh								4,8,17,29,34,51
	Gazelle								5,10,11,25,26,35,38
	Petkus	x		x		x		x	37,42,48
	Prolfic Spring								30,41,42,45,50
	Wren Abruzzi								5,23,34,36
	Extra Early Rye1								
	Somro								4,5,9,36
	R1210								21
	118 Baltia								4,7,9
	R797								4,19,25,27,32,41,44,53
	Fl-Synt								10,18,25,30,32,38
	445979 Ottowa Select								4,6,8,19,25,38,41,51
	Gulzow Kunz CT1			x		x			9,38
	Rogo								17,41,45,50,52,55
	Florida 401	x							7,10,40
	L-145-N					x		x	7,17,27,35,50,55
	L-145-P								20,21,22,23,24,27, 31,32,39,41,53,54,55

Anthocyanins

Cyanidin glucosides

A. Cyanidin 3,5-O-diglucoside
B. Cyanidin 3-O-rutinoside
C. Peonidin 3-O-rutinoside
D. Peonidin 3-sophoroside or Peonidin 3,5-diglucoside

Delphinidin glucosides

E. Delphinidin rutinoside
F. Delphinidin 3-o Glucoside
G. Delphinidin 3-glucoside

Pelargonidin glucosides

H. Pelargonidin 3-O-glucoside
I. Pelargonidin 3-sambubioside

Table 4.11: Uncharacterized compounds in rye grain

Compound	Formula	M/Z experimental
1	$C_{36}H_{35}O_9$	611.2281
2	$C_{43}H_{31}O_4$	611.2222
3	$C_{25}H_{39}O_{17}$	611.2187
4	$C_{28}H_{35}O_{15}$	611.1975
5	$C_{35}H_{31}O_9$	595.1968
6	$C_{28}H_{35}O_{14}$	595.2026
7	$C_{35}H_{31}O_{10}$	611.1917
8	$C_{27}H_{33}O_{13}$	565.1921
9	$C_{39}H_{31}O_7$	611.2069
10	$C_{42}H_{27}O_5$	611.1858
11	$C_{24}H_{35}O_{18}$	611.1823
12	$C_{21}H_{39}O_{20}$	611.2034
13	$C_{46}H_{27}O_2$	611.2011
14	$C_{32}H_{35}O_{12}$	611.2129
15	$C_{25}H_{34}O_9$	478.2202
16	$C_{38}H_{32}O_2$	520.2402
17	$C_{45}H_{23}O_3$	611.1647
18	$C_{34}H_{27}O_{10}$	595.1609
19	$C_{42}H_{27}O_4$	595.1909
20	$C_{34}H_{27}O_{11}$	611.1553
21	$C_{28}H_{16}O_{13}$	560.059
22	$C_{16}H_{35}O_{24}$	611.1518
23	$C_{38}H_{27}O_8$	611.1705
24	$C_{38}H_{27}O_7$	595.1756
25	$C_{31}H_{31}O_{13}$	611.1764
26	$C_{45}H_{24}O_3$	612.1719
27	$C_{34}H_{32}O_5$	520.2244
28	$C_{20}H_{35}O_{20}$	595.1716
29	$C_{31}H_{36}O_7$	520.2455
30	$C_{20}H_{35}O_{21}$	611.1665
31	$C_{25}H_{21}O_9$	465.1185
32	$C_{38}H_{28}O_8$	612.1784
33	$C_{31}H_{32}O_{13}$	612.1842
34	$C_{32}H_{30}O_4$	478.2144
35	$C_{21}H_{34}O_{12}$	478.205
36	$C_{24}H_{40}O_{12}$	520.2519
37	$C_{34}H_{28}O_{11}$	612.1631
38	$C_{27}H_{36}O_{10}$	520.2308
39	$C_{35}H_{15}O_5$	515.0919
40	$C_{35}H_{26}O_2$	478.1932
41	$C_{28}H_{30}O_7$	478.1991
42	$C_{30}H_{32}O_8$	520.2097
43	$C_{31}H_{31}O_{12}$	595.1815
44	$C_{37}H_{18}O_8$	590.1011

45	$C_{27}H_{32}O_{16}$	612.169
46	$C_{32}H_{17}O_{4}$	465.1126
47	$C_{30}H_{15}O_{11}$	551.0614
48	$C_{17}H_{34}O_{15}$	478.1897
49	$C_{15}H_{26}O_{24}$	590.0814
50	$C_{17}H_{23}O_{18}$	515.0884
51	$C_{20}H_{36}O_{21}$	612.1749
52	$C_{23}H_{36}O_{13}$	520.2155
53	$C_{33}H_{23}O_{4}$	483.1596
54	$C_{14}H_{25}O_{17}$	465.1091
55	$C_{32}H_{19}O_{6}$	499.1181

concentration with eight different anthocyanins, including a delphinidin and pelargonidin, while six were uncharacterized peaks.

In addition to the characterized anthocyanins, there were a large number (55) of compounds for which chemical formula could be predicted but precise identification of compounds could not be done (Table 4.11). Interestingly, the cultons with high anthocyanin concentrations had two to eight of these uncharacterized compounds. However, there was no significant trend to associate a specific compound with high anthocyanin concentration. These compounds could be of value to future researchers.

4.7. Impact of grain characteristics on germination frequency and WFS.

As presented in Figure 4.1, kernel-weight of seed used for seeding had a positive effect on field germination frequencies and also WFS scores to a lesser degree (R^2 = 0.205; Table 4.2). Thus, the correlation confirmed previous observations that higher seed weight contributes towards higher germination frequencies (Manonmani and Somasundram, 2014), which indirectly promotes higher WFS for winter cereals.

CHAPTER 5

Discussion

5.1. Specific compounds produced by winter cereals

The physiological properties of winter-hardy rye are due to the presence of several specialized metabolic processes and accumulation of specific biological compounds when exposed to low temperatures (Guy *et al.,* 2008). An extended period of cold exposure during autumn triggers a number of metabolic processes to improve the tolerance of the plant to harsher winter conditions (Thomashow, 1999; Janmohammadi *et al.,* 2018). Among the specific compounds produced and identified in winter cereals during cold acclimation are anti-freeze protein, betaine, sugars, polyamines, and certain anthocyanin compounds, which all are postulated to reduce or hinder the effects of frost-induced damage to plant tissues during winter (Griffith and Yaish, 2004; Guy *et al.,* 2008; Nadeau *et al.,* 1987; Thomashow, 1999; 2010; Cao et al 2021). The anthocyanin compounds were of special interest in this study as they, in contrast to anti-freeze proteins and betaine, are also produced in seeds. Thus, the study was aimed at determining if winter hardiness levels in winter rye could be predicted based on the anthocyanin composition or other extractable compounds in seeds. During the study, the question was raised if the growth conditions for seeds used for seeding had any significant effect on WFS.

5.2. Differences between greenhouse and field grown rye

Data for the study was collected on a number of different plant and seed qualities from rye cultons grown in two environments, greenhouse and field. When the two data sets were compared though, there were some interesting findings to be seen, even before performing statistical analyses. One obvious difference was the green house plants were overall taller, had longer spikes and longer awns (Figures 4.4 and 4.5). These types of traits often show pleiotropic effects on each other and depend on a higher cell elongation (McKim 2019; Peng *et al*., 1999) in stems, spikes, and awns. Seeds from the greenhouse had lower starch content, higher protein content, and were narrower than grain from the field. The seed morphology and a high

protein/starch ratio suggested the greenhouse conditions were more stressful during grain filling than grain matured in the field. Less available carbohydrates for allocation to seeds, a lower transport of photosynthate products to the seeds, and/or a shortening of the grain filling period could cause poor grain filling (Jaiswal *et al*., 2020).

The greenhouse grown rye were as the name states, grown mainly under static greenhouse conditions. There was an initial period in which the conditions of an autumn/winter environment were simulated in the phytotron growth chamber, but that was only for the initial germination and growth phase of the rye. The remainder of the lifespan of this group of rye was spent under a continuous greenhouse environment with regular watering, quite different from the conditions out in the field. In contrast to the phytotron environment, the field grown rye may have experienced short exposures of cold prior to a longer period of cold and/or a second hardening step at sub-zero temperature, which are conditions that promote higher freezing tolerance in plants (Leuendorf *et al.,* 2020; Zuther *et al.,* 2019). Also the exposure to a longer period of low temperatures, and more temperature and light fluctuations than the greenhouse grown rye would have promoted a higher freezing tolerance (Huner *et al*., 2016; Mayer *et al*., 2020;); Subsequently, the degree in which the cold tolerance mechanisms of the rye were engaged, could be a cause for the variation observed in the data and may have impacted the later stages of the plant development, and possibly contributed to the differences observed in this study.

Another potential cause for the variation observed would be what the rye was grown in. The greenhouse rye was sown and organized differently from those plants sown in the field. Each culton in the greenhouse group had ten seedlings initially planted, with each one being in a self-contained plant pot with potting soil. In contrast, the field rye was organized into rows, with 100 plants per culton being sown into open ground. The potted plants were kept on raised mesh tables, which increased the evaporation rate and temperature exposure of the soil in them, when compared to the more insulated conditions of the soil out in the field. These differences in temperature, soil quality, soil water holding capacity, and available space for root development are all potential causes for the differences in the results between the field and greenhouse grown rye groups.

The intensity of light received by the rye plants may have been another factor for the observed differences between the two rye groups. The greenhouse grown rye never had access to unfiltered sunlight throughout their life span. In the phytotron growth chamber for their initial germination and cold hardening phase, all light was provided through artificial means. Once the more mature rye had been moved to the greenhouse, natural light was available, but it was filtered through both the glass of the greenhouse, and later when the pollen had begun appearing through the white pollen netting as well. In contrast, the field grown rye did not have artificial lighting at all during the complete lifespan, and while they did have pollen nets place over them, there was nothing else except cloud cover to impact the quantity and intensity of the light reaching them.

There are several conclusions which may be drawn from the differences observed between the two environmental groups. The relatively short and mild cold acclimation period, followed by an extended growth period in a warm, stable greenhouse environment encouraged much greater growth of the rye plant itself as compared to the harsher field conditions. The length of the grain filling period may have been shortened by the greenhouse growth conditions. The different growth environments appear to have also influenced the composition of the rye grain as well. The field grown rye grain appears to overall have a greater concentration of anthocyanin content, a higher starch content, and a lower protein content than the greenhouse grown rye grain. Finally, the color index of the rye grain showed some variation as well, with greenhouse grown rye grain having slightly higher color values than field rye grain. This could indicate that the environmental variation between these two groups affected the metabolic process of grain formation in the rye plant, contributing to the different composition of specific compounds observed in the analysis.

One objective of this study was upon the winter hardiness level of different rye cultons, and to study if WFS levels were associated with any plant or seed characteristic. Organizing the data into a number of boxplot analysis helped to see which of the rye physical attributes and phenotypic characteristics recorded were affected the most by the winter hardiness level of a rye line. There were several characteristics which displayed notable variation between the different winter hardiness levels, such as rye spike length, awn length, and grain weight. The

range of variation between the winter hardiness categories differed between environmental groups for some rye characteristics. Grain weight had a greater range of variation between different winter hardiness categories in greenhouse grown rye than in field grown rye. This could potentially be due to the stable environment of the greenhouse resulting in fewer of the plants dying due to environmental stressors, therefore allowing for outlier individuals to survive and produce a yield.

A major focus in the study was to investigate if any anthocyanin compound in the seed could be associated with the level of winter hardiness. This investigation was prompted by the finding that cyanins in cold-acclimated leaves and crown tissues are associated with higher WFS (Bahrani et al 2019). An analysis of color pigments in seeds revealed a slight tendency towards darker and lighter yellow seeds from the two lowest WFS classes grown in the field, but the color differences between WFS classes and environments were small. The anthocyanin concentrations varied more significantly in the greenhouse grown rye than the field grown rye, although the mean values of these two groups were very similar to each other, 3.1 and 3.01 μg mL^{-1}. While the maximum value of 10.7 μg mL^{-1} was an anomaly present in only one line, L-286-R, other lines such as Dakota and Reimann Philipp had values exceeding 6 μg mL^{-1}, greater than the maximum values we observed in the field rye. A total of 21 greenhouse grown rye had low anthocyanin concentrations less than 2, while the field grown rye having 12, close to half that number of low concentration lines. The cause for the differences between the two groups is possibly due to the unique environmental conditions experienced by each set of winter rye.

The variation in anthocyanin concentration ranges and other observed rye qualities between these two growing groups is both a potential source of error in these analyses, and a means to compare the impact different growing environments had upon the rye. The mass-spectrometer specific anthocyanin determination analysis saw a noteworthy difference between the two groups. Though as seen in Tables 4.5 and 4.6, the correlation analysis done using total anthocyanin concentrations observed different significant relations be present in each group.

5.3. Significant relationships present in winter rye lines

5.3.1. Phenotypic characteristic correlations

The results of the ANOVA analysis upon the rye samples have identified significant relationships between certain phenotypic factors of the rye cultons employed in this study, such as hundred grain weight and plant height at harvest, and the total anthocyanin content of the rye grains (Appendix A, Table A1 and A2). This indicates both that some of the phenotypic characteristics of a rye grain could be used as an estimate for total anthocyanin rye content, and that some of the phenotypic characteristics of rye can potentially be associated with anthocyanin compounds. On their own, these results do not completely validate the stated hypothesis of this study, but it does support the notion that certain characteristics of winter rye may show pleiotropic effects on the presence and concentration of anthocyanin compounds in grain. This can in turn serve as potentially supporting evidence for the hypothesis, since if anthocyanin content has a significant relationship with one characteristic of rye, it is possible that they may be connected to another.

The relationships identified from the correlation analysis have provided evidence to both support and detract from the stated hypothesis (Tables 4.2, 4.3, 4.5, 4.6). The WFS BLUE winter hardiness value of the selected rye lines was found to have highly significant relationships ($p < 0.001$, $p < 0.01$) with several of the other rye variables which were measured in this study. However, there was no significance found with the total anthocyanin concentration values of the rye grain (Tables 4.5 and 4.6). The strength of these relationships is not consistent between the various rye characteristics. The relationship between WFS BLUE and rye spike length was highly significant. The R-value of that relationship was 0.262, which indicates that 26.2% of the totality of these two variables are what possesses that highly significant relationship. Other rye grain characteristics which had significant relationships with WFS included external phenotypic characteristics including grain length and grain weight, along with composition characteristics like starch content and protein content of the rye grain. While the results of this and other significant relationships with winter hardiness are not something to be dismissed, the apparent strength of this relationship is not absolute either. This does not necessarily mean that this disproves the hypothesis though, merely that it cannot be said with absolute certainty that it does prove it.

The results of the correlation analysis did not find many significant relationships for the total anthocyanin concentration values. Of the different rye characteristics analyzed, only stem height, protein percentage, and the mean b^* value from the color analysis were found to have a significant relationship with total anthocyanin concentration. The lack of a statistically significant correlation with winter hardiness is a point against the hypothesis. The minor significance ($p < 0.05$) between anthocyanins and proteins, however, may be a possible avenue to explore, given the significant correlation ($p < 0.01$) between WFS and protein concentration.

5.3.2 Specific anthocyanin compound findings

The mass-spectrometer analysis found a wide range of potential individual anthocyanin compounds in the rye grains. While many of the specific anthocyanin compounds found were present only in a single rye culton, several of these compounds were observed in multiple rye cultons. Some compounds like $C_{38}H_{27}O_8$ were identified in both greenhouse and field grown rye, but the majority were only observed in one of the two rye growth groups. The number of compounds identified for each culton varied significantly between the different winter hardiness categories for the greenhouse grown rye ($p < 0.01$), with Very Low and Very High having the high numbers of anthocyanins. The same variation was present to a lesser degree for the field grown rye, with Low WFS cultons having the most compounds identified ($p < 0.1$). Of these multiple line compounds, a portion of them were found in higher proportions for certain winter hardiness categories. An example of this would be $C_{45}H_{24}O_3$, which out of all the rye cultons that we observed it in, 42.8% of them were categorized as very high in terms of winter hardiness, three times more than any other winter hardiness category in which this compound was observed in. Combining this information with knowledge of the winter hardiness ratings of all 96 different cultons of winter rye, it was possible to identify any trends in how often these compounds were present in rye of a certain rating. Based on the current findings, several compounds have been identified that may serve as an indicator of a specific rating of winter hardiness if found present in a rye culton. This is not a certainty, since many of these compounds of interest were observed in rye cultons from every winter hardiness category. An example of this would be the compound $C_{34}H_{32}O_5$, as a minimum of ten percent of the total number of observations of this compound

occurred in a winter rye culton from each of the five ratings of winter hardiness used in this study. It should also be noted that many of the anthocyanin readings taken from these rye grain extracts were notably weak, with a strength rarely rising above 100 mhz. This phenomenon occurred more frequently in the greenhouse rye samples than the field ones. These weak readings made it difficult to detect any potential anthocyanin compounds, and it is quite possible that there were potential anthocyanin compounds which were not detected or overlooked due to their weak signals. While some potential compound compositions were able to be determined from these readings regardless, they are still a problematic factor to come to a strong conclusion.

While the weak MS readings exhibited by some rye samples does increase the possibility of there being compounds present in these samples that have gone undetected, the data from the overall MS analysis is still a valuable source of information. One example of the potential value of these findings can be observed with $C_{28}H_{30}O_7$, which was identified as being present in 16 of the field grown rye cultons (Table 4.11). Of these 16 rye cultons, 9 of them, or 56%, were classified as being in the “Low” and “Very Low” winter hardiness categories, with only 25% being classified as “High” and “Very High”. This suggests that the presence of this compound in a rye culton could be evidence of a probable low tolerance to winter conditions. Having this knowledge would likely aid in providing the individual or group cultivating that rye line with the information required to make a reasonable estimate of the winter hardiness of their rye.

The findings of the MS-MS chromatogram appear to indicate a link between the results of this study and the findings of Bahrani et al (2019). While the initial mass spec analysis found a couple of the compounds previously identified in leaf and crown tissue, a number of the compounds observed in the selected rye cultons were not able to be identified beyond their suggested molecular formula. Running this additional method on some of the field rye samples helped to confirm the presence of several more specific anthocyanin compounds being present in rye grain.

5.4. Completion of stated objectives

The overall results of this study on winter rye indicate that certain attributes of a winter rye culton can serve as a potential marker for specific characteristics of that culton, such as winter hardiness. These attributes can include the specific phenotypic qualities of a rye grain or the presence of certain anthocyanin compounds in that grain. The results of the various analysis have provided a quantity of intriguing data, along with helping in meeting the stated objectives for this project.

Of the stated objectives, the first of them, determining total and specific anthocyanin content in the panel of rye lines, appears to have been a success. The total anthocyanin concentration value for all the rye lines have been successfully recorded, though it should be noted that for the field grown rye, the lack of available material for several lines due to losses from environmental and biotic factors such as the ergot meant that they were not included in that dataset. For determining the presence of specific anthocyanin compounds in the rye grain, as previously mentioned the MS analysis was able to identify a number of different candidates from the rye samples. The presence of these compounds, along with the frequency at which they occur in rye cultons of different winter hardiness categories, may fulfil not only that aspect of the first objective, but also the fourth. With a list of specific compounds found in these rye lines, observing trends that arise from their distribution can give an idea of what winter hardiness categories a compound may be more likely to be present. As previously mentioned, this information would be of potential value to anyone seeking to predict those characteristics in a rye culton.

For the second and third objectives, the various analyses undertaken have provided the necessary information required to meet them. The results of the ANOVA and correlation analyses offered mixed support the possibility of a statistically significant link existing between winter hardiness and anthocyanin compounds in winter rye. The ANOVA analysis found some relationships between total anthocyanin and WFS values, while the correlation analysis did not find any significant relationships between the two factors in the selected rye cultons. It would appear that based on these findings, there is not a significant direct relationship between the total anthocyanin concentrations present in rye grain, and the winter hardiness levels of that

particular rye culton. The relationship between anthocyanin content, grain weight, and color values were more fortunate in that regard. Both the ANOVA and the correlation analysis found significant relationships between anthocyanin content and some of the color data values collected. The ANOVA also observed a significant relationship between grain weight and anthocyanin content, though this significance was not found for the correlation between the two factors. This data indicates that while there are aspects of rye grain in which total anthocyanin content is a significant factor, it does not appear that winter hardiness values like WFS or LT_{50} are among them.

5.5. Conclusions

The main objective was to determine if the presence of specific anthocyanin compounds in rye grain can serve as an indicator of the winter hardiness classification for that specific rye culton. While the results of this study have not provided an exhaustive list of specific compounds linked to different winter hardness levels, additional data might provide a more definitive answer to the question of grain-specific anthocyanin's viability as an accurate predictive marker for winter hardiness. From these results it may also be concluded that the physical characteristics of the grain such as grain length, width and weight, are another potential indicator for the winter hardiness classification of a rye line. Some other factors of rye grain composition, such as starch or protein content are both potential markers for winter hardiness as well, based on the significance of their relation to WFS in this study. The importance of grain physical and compositional characteristics in predicting other qualities of rye, or in predicting winter hardiness in other cereals, may be an avenue for future endeavors in this field of study. Further research on the frequency of occurrence and end usage of grain-specific anthocyanin compounds in winter rye, as well as identifying additional compounds or phenotypic attributes of rye which have a strong affinity to a certain level of winter hardiness classification, may prove valuable for confirmation and refinement of this conclusion.

5.6. Future research

The study has revealed some interesting results, especially in relation to characterization of anthocyanin compounds. A previous study (Bahrani *et al*. 2019) had revealed the presence of cyanidin glycosides in leaf and crown tissue with WFS, however, only a few of these compounds were detected in the grains. However, an additional 55 new compounds were found in the rye grain extracts. Further study needs to be done to characterize the compounds and study if these compounds are associated with WFS. An additional potential advantage to explore will be to find out if any of these compounds can add to the health benefits of rye grain. Research into the construction of a more complex predictive model for rye WFS to include all factors of importance is another possible avenue of study.

CHAPTER 6

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Appendix A:

Table A1. ANOVA analysis of greenhouse rye for total anthocyanin concentration and rye phenotypic factors: grain width and length, average plant height at harvest, head length, awn length, hundred grain weight. (significant relationships only, all others non-significant.)

Greenhouse ANOVA for total anthocyanin	P-value
Grain length	0.05
Average height	0.001
Grain length: Grain width	0.1
Hundred grainweight : Average height	0.01
Hundred grain weight: Average head length	0.05
Hundred grain weight: One grain length: Average head length	0.1
Hundred grain weight: Average height: Average head length	0.1
Hundred grain weight: One grain width: Average height: Average head length	0.05

Table A2. ANOVA analysis of field rye for total anthocyanin concentration and rye phenotypic factors: grain width and length, average plant height at harvest, head length, awn length, hundred grain weight. (significant relationships only, all others non-significant.)

Field rye ANOVA for total anthocyanin	P-value
Hundred grain weight	0.05
One grain width: Average height	0.05
Hundred grain weight: One grain width: Head length	0.1
Hundred grain weight: One grain length: Average height	0.1
Hundred grain weight: One grain length: One grain width: Awn length	0.05