THE IMPACT OF NITROGEN FERTILIZATION ON THE PHYSICO-CHEMICAL, FUNCTIONAL, AMINO ACID PROFILE, PROTEIN SECONDARY STRUCTURE AND BREAD PROPERTIES OF INTERMEDIATE WHEATGRASS.

A Thesis

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Dedication

I dedicate this thesis to my parents, Margaret Dei Aduama and Fredrick Aduama, whose unwavering love and selfless sacrifices for their children have shaped my path. Although they are no longer with me, this work stands as a tribute to their constant support and enduring affection. I also dedicate this thesis to my wife, Frances Delali Aduama, whose unwavering love and steadfast support have been my foundation throughout my master's journey.

Abstract

Intermediate wheatgrass (IWG), scientifically known as *Thinopyrum intermedium*, is a cool-season sod-forming perennial grass belonging to the *Triticeae* tribe of the *Pooideae* subfamily. Compared to its cousin wheat, IWG has great potential for ecosystem services such as reduced soil erosion, lowered nutrient leaching to groundwater, and improved carbon sequestration. It has higher protein, dietary fiber, and ash. It is one of several perennial crops available for potential food use and the first commercially viable perennial grain in the United States. Lack of adoption of perennial grains for food use is essentially attributed to grain yield shortfall compared to annual grain crops. The dual use of IWG for food and feed is therefore crucial to their adoption. As part of efforts to develop IWG for food application, agronomists are currently exploring application of nitrogen fertilizer to the crop. This study investigated the application of nitrogen fertilizer (80 lbs./acre) in the spring or fall in Minnesota and Wisconsin affects: (1) the physico-chemical and functional properties of IWG; (2) the amino acid profile, protein secondary structure and the bread properties of IWG. Refined and wholegrain samples were prepared for analysis. Nitrogen treatment increased protein and fat content of IWG while reducing total carbohydrates, regardless of location, treatment time or refinement. Total dietary fiber increased in whole grain samples. Starch hot paste viscosity generally decreased with nitrogen treatment. Farinograph water absorption and dough stability increased with nitrogen fertilization in samples from Wisconsin. IWG kernel size increased with nitrogen application regardless of growing location and timing of treatment. Nitrogen fertilizer application in the spring increased amino acid content in refined and whole grain intermediate wheatgrass samples from Minnesota and Wisconsin. Spring-treated refined samples exhibited significant increases in amino acid concentrations, specifically arginine,

serine, leucine, phenylalanine, and tyrosine in Minnesota, and serine in Wisconsin, whereas fall treatment demonstrated no significant effect. In Minnesota whole grain samples, amino acids except alanine and proline increased significantly. Nitrogen treatment also affected protein structure, increasing β-sheets and decreasing β-turns in refined samples, with the opposite observed in whole grains, except for fall-treated samples from Wisconsin. Loaf height increased in refined samples from Minnesota, although loaf volume remained unaffected. Nitrogen also increased bread firmness in refined samples but decreased it in whole grains. Cell count dropped in whole grain bread with nitrogen treatment, except for an increase in fall-treated samples from Wisconsin.

Key words: Intermediate wheatgrass, nitrogen fertilizer, physico-chemical, amino acid profile, protein secondary structure, bread properties.

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1: LITERATURE REVIEW

1.1: Introduction

The dominance of annual cereal grains in both human diets and global agriculture has indeed been significant, constituting approximately 50% of human calorie intake and agricultural land use worldwide. However, this high reliance on annual grains has contributed to environmental degradation such as soil erosion, loss of biodiversity, and greenhouse gas emissions. The Food and Agriculture Organization (FAO) highlighted in 2016 the increasingly alarming impact of climate change on agriculture, emphasizing its detrimental effects on food security. Climate change-related factors such as extreme weather events, shifts in precipitation patterns, and rising temperatures pose significant challenges to crop production systems worldwide. Thus, there is a pressing need to find alternative grains to traditional annual grains. Perennial grains, for instance, have the potential to provide sustainable food production while mitigating some of the negative environmental impacts associated with annual cropping systems (Jungers et al., 2017). Efforts to develop and promote perennial grains as viable alternatives to annual grains are underway globally. By harnessing advancements in agricultural technology, genetics, and agronomic practices, researchers and farmers seek to create cropping systems that are more resilient to climate change, require fewer inputs, and promote ecosystem health (Jungers et al., 2017). The shift towards perennial grain agriculture represents a significant paradigm change in food production systems. By incorporating perennial crops into agricultural landscapes, we can promote biodiversity, conserve natural resources, and build resilience to climate change. Moreover, the ability of perennial grains to provide food directly for human consumption contributes to food security by diversifying our food sources and reducing our reliance on annual crops. Intermediate wheatgrass

(IWG), scientifically known as *Thinopyrum intermedium*, is a cool-season sod-forming perennial grass. It belongs to the *Triticeae* tribe of the *Pooideae* subfamily (Kantarski et al., 2018). Originally native to central Europe, the Balkans, and Asia Minor, IWG was introduced to the United States in 1932 primarily as a forage crop. Since then, it has been cultivated extensively throughout the western regions of North America (Hybner & Jacobs, 2012). Trials and research on IWG have demonstrated its potential as a multi-functional crop, capable of yielding various commodities while providing valuable ecosystem services. Unlike annual crops such as wheat, which often deplete soil organic matter and require substantial inputs, IWG offers a more sustainable alternative. One notable advantage of IWG is its ability to yield crops while simultaneously building soil organic matter, thus enhancing soil health and fertility over time (Culman et al., 2013). This characteristic makes IWG particularly valuable in sustainable agriculture systems, where maintaining soil quality and resilience is important. By promoting soil health and organic matter accumulation, IWG contributes to improved soil structure, water retention, and nutrient cycling, ultimately enhancing the overall productivity and sustainability of agricultural lands. The exploration of nitrogen fertilizer application in Intermediate wheatgrass (IWG) cultivation is a significant aspect of efforts to develop it for mainstream food use. Nitrogen plays a crucial role in plant growth and development, and optimizing its application can have substantial impacts on crop yield, quality, and nutritional content (Dobbratz et al., 2023). Since the mid -20th century, farmers have significantly increased the application of nitrogen fertilizer in cereal crops as an agronomic practice aimed at boosting productivity (Hawkesford, 2014). The global demand for nitrogen fertilizer utilization is expected to reach 111.6 tons in 2022 (FAO, 2019). Despite the agronomic and nutritional benefits of applying nitrogen fertilizer to meet food security needs through

increased grain production (Smil, 1999; Swarbreck et al., 2019), its use is under growing scrutiny due to greenhouse gas emissions associated with nitrogen fertilizer volatilization (McIsaac et al., 2010). Consequently, there has been a push towards innovation (Jez et al., 2016; Yan et al., 2020) and the establishment of policy (Environment and climate change Canada, 2020) targets aimed at reducing nitrogen application to crops (Trevisan et al., 2022). To achieve these goals, optimum nitrogen fertilizer application and the time it is applied have been the major strategies to improve crop yield, quality and nutritional content (Shejbalova et al, 2014).

Nitrogen is a limiting nutrient for cereal production and IWG grain yield (Jungers et al., 2017). The application of optimum nitrogen is beneficial to the grain yield of IWG (Jungers et al., 2017). The stand against nitrogen management globally is to come up with nitrogen to meet global food security while reducing the flow of excess nitrogen to the environment (Ladha et al., 2020). To address such a challenge, one key approach is to enhance nitrogen use efficiency (NUE) which not only improves crop yield but also reduces environmental losses through assiduous agronomic management, and measures to improve soil quality over time (Ladha et al., 2020). Nitrogen has a positive effect on amino acid content (Liu et al., 2010) and grain protein accumulation in cereals, with nitrogen top dressing at the panicle development stage playing a major role in brown rice (Ning et al., 2010). Optimum nitrogen fertilizer application is shown to enhance the protein quality of crops by stimulating amino acids and protein synthesis (Liu et al., 2022).

Intermediate wheatgrass is nutritionally similar to common wheat but contains a slightly higher protein content and lower gluten levels (Becker et al., 1986). While the protein in IWG grain is richer than that of wheat, it is, like other grains, limited in lysine. However, it boasts a higher proportion of other essential amino acids compared to wheat (Becker et al., 1991). IWG flour

exhibits the properties of a thick, viscous dough suitable for denser baked goods such as muffins or pancakes. Baked goods made solely from IWG flour, like muffins and cookies, were rated good to excellent overall. However, dough made from the endosperm flour of IWG variety showed weak viscoelastic properties (Zhang et al., 2015). Bread-making quality is influenced largely by the gluten proteins, gliadin and glutenin, that form a viscoelastic dough. This dough has gas retention ability required to produce loaves with large volumes and desired texture (Flæte et al., 2005). Liu et al., 2022, showed that nitrogen treatment increased the beta sheet content in wheat, improving the rheological properties of wheat dough and bread making qualities.

As part of efforts to develop IWG for food application, agronomists are currently exploring the application of nitrogen fertilizer to the crop. To make perennial grain and biomass crops both profitable and environmentally sustainable, it is essential to establish optimal nitrogen fertilizer application rates (Cerrato and Blackmer, 1990; Randall and Mulla, 2001). Nitrogen is a key limiting nutrient for cereal crops, including intermediate wheatgrass (IWG), influencing both its grain yield and overall productivity (Jungers et al., 2017). Recent studies indicate that nitrogen's role in IWG grain production is more about enhancing plant development, which in turn increases the potential for higher grain yields, rather than directly influencing the grain itself (Fagnant et al., 2023). Applying optimal levels of nitrogen has been shown to improve IWG grain yield (Jungers et al., 2017). A recent report also found that a nitrogen application of 60 kg N ha^{-1} resulted in higher grain yields and lower biomass production compared to higher nitrogen rates or no nitrogen, suggesting that a balanced approach between grain production and plant growth is key (Mulla et al., 2023).

Previous research on IWG has focused on the chemical characterization and functionality of IWG grain (Rahardjo et al., 2018) and the impact of the rates and timing of nitrogen fertilizer treatment on forage yield and grain size of IWG (Jungers et al., 2017). However, little is known about the influence of nitrogen fertilization and timing on the end use characteristics of IWG grains. The purpose of this study is to evaluate the impact of nitrogen treatment in the spring and fall season on the nutritional and bread properties of IWG grown in Minnesota and Wisconsin. This is an important final step in the development of IWG for food use.

Our objectives are as follows:

To evaluate the effect of nitrogen treatment on the physico-chemical and functional properties IWG grains harvested from different locations.

To investigate how optimum nitrogen fertilization for IWG affects the amino acid profile, protein secondary structure, and bread properties of the crop.

1.2: Origin and domestication of intermediate wheatgrass

Intermediate wheatgrass (IWG), scientifically known as *Thinopyrum intermedium*, is a cool-season sod-forming perennial grass. It belongs to the *Triticeae* tribe of the *Pooideae* subfamily (Kantarski et al., 2018). Originally native to central Europe, the Balkans, and Asia Minor, IWG was introduced to the United States in 1932 primarily as a forage crop. Since then, it has been cultivated extensively throughout the western regions of North America (Hybner & Jacobs, 2012). Inspired by Wes Jackson's vision (1980) for a diverse, herbaceous perennial polyculture, the Rodale Research Center (now Rodale Institute [RI]) began evaluating around 300 species in the early 1980s for their potential in perennial agriculture, mimicking the structure of natural ecosystems. After assessing more than 100 grass species, intermediate wheatgrass (IWG,

Thinopyrum intermedium (Host) Barkworth & D.R. Dewey) was chosen in 1985 for its promise as a perennial grain crop. It was selected based on its favorable plant structure, seed production traits, perennial growth, potential for food use (Wagoner 1990a), and its genetic relation to important *Triticeae* grain crops (Wagoner 1990b), which suggested a good nutritional profile and absence of anti-nutritional compounds. Before being considered for domestication, IWG had a long history of use in the United States for erosion control, forage production (Asay and Jensen 1996), and as a secondary gene pool for wheat improvement (Li and Wang 2009; Pototskaya et al. 2022). In the 1930s, scientists had high hopes for wide hybridization, particularly when N.V. Tsitsin in the Soviet Union, along with researchers in the USA and Canada, began experimenting with hybridizing bread wheat and intermediate wheatgrass (IWG) to develop perennial wheat varieties (Suneson et al., 1963; Tsitsin, 1978).

In the 1980s, the Rodale Research Center (Kutztown, USA) selected intermediate wheatgrass (IWG) from over 100 perennial species for domestication and seed production. Among perennial crops, IWG stands out for its relatively large seeds, moderate spike fragility, good threshability, high biomass, and excellent fodder quality (Wagoner, 1990; Becker et al., 1992). Two selection cycles focused on improving agronomic traits and seed size were completed, leading to the identification of promising wheatgrass genotypes (clones). These selected genotypes were then transferred to the Land Institute (Salina, Kansas, USA) for further research and development (DeHaan et al., 2005; Cox et al., 2010).

At the Land Institute, selection cycles began with the development of indices based on key traits, such as seed weight per plant, seed weight per spike, percentage of bare seed, thousand kernel weight, and disease resistance. For each selection cycle, a population of 50–70 genotypes with the

most favorable combination of these traits were selected for over-pollination. After two cycles of selection, grain yield per unit area increased by 77%, and seed weight grew by 23% (DeHaan et al., 2018). At both the Land Institute and the University of Minnesota (Minnesota, USA), genome sequencing data (*Thinopyrum intermedium* v2.1 DOE-JGI, https://phytozome-next.jgi.doe.gov/info/Tintermedium_v2_1) became an important tool in the domestication of *T. intermedium*. This enabled the use of genome-wide association studies (GWAS) and bioinformatics methods to accelerate the selection process, replacing more time-consuming phenotype-based approaches (Bajgain et al., 2019; Crain et al., 2020, 2021).

After many years of research at the Land Institute, the wheatgrass variety Kernza was developed, named after the residents of Kansas. This variety is used for seed production, as well as for forage and hay (haylage). In the second year of cultivation, Kernza demonstrated significant environmental benefits, including an 86% reduction in nitrate leaching into groundwater and a 13% increase in soil carbon sequestration compared to annual crops (Glover et al., 2010; Culman et al., 2013; DeHaan, Van Tassel, 2014; Pugliese et al., 2019). Kernza is highly resistant to diseases and pests, requiring fewer agricultural inputs such as nitrogen fertilizers, tillage, pre-sowing seed treatments, and fungicide applications. This results in lower energy and economic costs (DeHaan et al., 2005; Pugliese et al., 2019).

In 2011, a joint breeding program between the Land Institute and the University of Minnesota was launched to improve Kernza, sparking commercial interest in this perennial cereal. The program focused on developing the MN-Clearwater variety, a synthetic population aimed at grain production, though it can also be used for biomass and forage. Seven parent genotypes were selected from 2,560 IWG genotypes based on traits like days to heading, plant height, spike weight,

seed size, and biomass production. In trials across Minnesota, MN-Clearwater produced 696 kg/ha, with a thousand-kernel weight of 6 g. The variety is characterized by a short stem (113 cm), good threshability (63%), and low stem fragility, showing minimal lodging. Domestication and improvement efforts for traits like seed size, threshability, spike fragility, and plant height are also underway at institutions including the University of Manitoba, the University of Utah, and the University of Agricultural Sciences (Uppsala, Sweden) (DeHaan et al., 2016).

Figure 1:Kernza with hull removed (i.e., dehulled) and grain-bearing spikes prior to threshing (photo credit: Alicia DeHaan)

1.3: Chemical composition of intermediate wheatgrass

1.3.1: **Protein content**

Intermediate wheatgrass has a significantly higher amount of seed storage proteins compared to common wheat. In a study of 60 genotypes, protein content in intermediate wheatgrass ranged from 16.4% to 23.6%, far exceeding that of a high-quality wheat line, which had 13.6% protein (Table 1). The total protein content was divided into SDS-extractable proteins (EP) and SDS-unextractable proteins (UP). SDS-extractable proteins were mostly monomeric or small polymeric proteins, while SDS-unextractable proteins typically consisted of larger protein polymers. In intermediate wheatgrass, SDS-extractable proteins made up most of the seed proteins, with an average content of 16.5%, while SDS-unextractable proteins averaged only 3.0%. The high

concentration of SDS-extractable proteins was the primary factor contributing to the elevated protein levels in intermediate wheatgrass. In genotype WG113104, the SDS-extractable proteins (19.5%) were nearly double the amount found in wheat (9.2%) (Zhang et al., 2015)

Table 1: Size-exclusion HPLC values of IWG and wheat samples

Empty Cell			IWG			Wheat	
			Mean	Median	Range	Poor	Good
SE-HPLC protein parameters	Protein content (%)		19.4	19.3	16.4–23.6	13.5	13.6
	%Whole flour	EP (%)	16.5	16.4	14.1–19.5	10	9.2
		UP (%)	3.0	3.0	1.5–6.0	3.6	4.4
		PPF (%)	1.4	1.3	0.5–3.5	1.8	2.3
	%Total proteins	EP%	84.9	85.5	74.9–90.8	73.6	67.5
		UP%	15.1	14.5	9.2–25.1	26.4	32.5
		PPF%	7.0	6.6	3.3–14.9	13.4	17.0

1.3.2: Protein composition in intermediate wheatgrass

While IWG's protein content is relatively high, it might not constitute appreciable amounts of the functional gluten-forming proteins (Wieser, 2007), gliadins and glutenins, but may have more albumins and globulins, given that these constituents are mostly found in the aleurone and germ (Tyl & Ismail, 2019). IWG glutenin proteins contain fewer high-molecular-weight glutenin subunits (HMW-GS) compared to wheat. While these subunits share a similar structure to wheat HMW-GS (67–120 kDa), they have a lower molecular weight, ranging from 45 to 90 kDa (Zhang X. et al., 2014). The reduced presence of HMW-GS with a molecular weight greater than 60 kDa in wheatgrass grains results in weaker gas-holding capacity and less dough elasticity, which contributes to lower bread-making quality (Marti et al., 2016).

1.3.3: Carbohydrate profile of intermediate wheatgrass

The carbohydrate composition of intermediate wheatgrass (IWG) differs significantly from that of wheat (Craine & DeHaan, 2024). Due to the smaller size of IWG seeds, they contain much less starch (46.7%) compared to wheat (72%) (Pototskaya et al., 2022). As endosperm size increases, the starch content tends to rise (Becker et al., 1991; Rahardjo et al., 2018). Marti et al. (2015) found that IWG flour had a lower starch content (46.7%) than refined wheat flour (73.9%) and whole wheat flour (72%). Similar findings were reported by Rahardjo et al. (2018) and Tyl & Ismail (2018), with starch content in IWG ranging from 46.74% to 52.45% and 42.53% to 52.96%, respectively. Starch consists of two main components: amylose and amylopectin. Amylose is a linear polymer of glucose units linked by α-1,4 glycosidic bonds (Tester, Karkalas, & Qi, 2004), while amylopectin is a branched polymer with a linear chain linked by α-1,4 glycosidic bonds, and branches connected by α-1,6 glycosidic linkages (Tester, Karkalas, & Qi, 2004). Rahardjo et al. (2018) reported amylose content in IWG whole flour ranging from 22% to 25%, while Tyl & Ismail (2018) found amylose content to be between 19.9% and 27.3% in the 2012 harvest. Amylose content is thought to influence bread firmness during baking, while amylopectin may contribute to bread firmness due to retrogradation during storage (Alcázar-Alay & Meireles, 2015).

Dietary fiber has been a dietary component of human health concern in the United States since 2005 (McGuire, 2011). It is made up of complex polysaccharides presented in plant tissues. It is not digestible by the human small intestine but can be fermented in the human large intestine (AACCI 2001). Resistant starch can also be classified as dietary fiber because it cannot be digested in the human small intestine but can be fermented in the large intestine (Slavin, 2013). The two major categories of dietary fiber are soluble and insoluble dietary fiber. Soluble dietary fiber

includes water-soluble arabinoxylans, β-glucan, fructans, and pectin. Insoluble dietary fiber includes water- insoluble arabinoxylans, cellulose, lignin, and resistant starch (Gebruers et al., 2008). Intermediate wheatgrass (IWG) has a significantly higher bran-to-endosperm ratio compared to closely related, more commonly cultivated grains like wheat, rye, or barley (DeHaan & Ismail, 2017). As a result, IWG contains more insoluble and total dietary fiber, along with higher levels of certain phytochemicals, which are predominantly found in the bran layers. However, IWG has a lower starch content compared to wheat (Tyl & Ismail, 2019). Higher total dietary fiber content has been reported in IWG flour (16.4%) than in whole wheat flour (11%) (Marti et al., 2015). In the United States, more than 90% of women and 97% of men do not meet the recommended intakes for dietary fiber, where consumption is only approximately 50% of the recommended intake (HJ Thompson, 2021). This represents the "fiber gap" in the United States, which has been well documented (Quagliani, D., & Felt-Gunderson, P. ,2017). A study conducted by Craine & DeHaan (2024) indicates that consuming IWG in equal amounts to wheat would provide 129% more dietary fiber. This shows that IWG has the potential in closing the "fiber gap" as a routine part of diets. The high fiber content in IWG however comes with a downside, as the arabinoxylans, which make up a significant portion of its fiber, can compete with gluten for water. This competition can negatively impact bread volume (Kantar et al., 2016).

1.3.4: Lipid content of intermediate wheatgrass

Intermediate wheatgrass (IWG) has a higher total fat content (3.2% on a dry basis) compared to whole wheat flour (2.8%) (Tyl & Ismail, 2018). This increased fat content is particularly relevant when considering storage stability. Higher fat levels in IWG may make it more prone to rancidity during storage, as lipids can serve as substrates for enzymes like lipase and lipoxygenase, which

catalyze the hydrolysis and oxidation of fats, respectively (Galliard, 1994). Additionally, lipids are susceptible to autooxidation, a spontaneous non-enzymatic process that occurs in the presence of oxygen, leading to off-flavors and reduced sensory appeal, which can limit shelf life (Galliard, 1994). The fatty acids in IWG flour can also form complexes with amylose in starch, creating resistant starch that slows starch hydrolysis (Tufvesson & Eliasson, 2000). In a study of IWG's fatty acid profile, Mathiowetz (2018) found that IWG is rich in polyunsaturated linoleic and oleic acids, which may increase its susceptibility to hydrolytic and oxidative rancidity. However, IWG's high antioxidant activity may help slow the rate of oxidative rancidity during storage (Mathiowetz, 2018).

1.3.5: Ash content of intermediate wheatgrass

Rahardjo et al. (2018) found that the ash content of bulk and experimental lines of IWG flour ranged from 2.60% to 3.80%, while the ash content in wheat ranged from 1.80% to 2.20%. Similarly, Tyl & Ismail reported IWG ash content to be between 2.44% and 3.01%, compared to wheat's 1.80%. The higher ash content in IWG can be attributed to its increased bran-to-endosperm ratio, as minerals are more concentrated in the bran (Šramková et al., 2009).

1.3.6: Nitrogen fertilization of intermediate wheatgrass

Historically, much of the world's cropland was occupied by perennial polycultures of wild plant species. However, with the advent of the agricultural revolution, farming practices shifted toward intensified production methods, such as tillage and annual crop cultivation, which focused mainly on growing grain for human consumption and livestock feed (Dewar, 2007; Cox et al., 2010). This expansion of annual row-crop agriculture has contributed to environmental issues, including pollution of marine and freshwater ecosystems, higher greenhouse gas emissions, and the loss of

biodiversity (Foley et al., 2011). In response, perennial crops have been proposed as a sustainable alternative to annuals for grain production (Glover & Reganold, 2010). Unlike annual crops, perennials have deeper root systems and longer growing seasons, which allow them to more effectively capture precipitation, utilize excess soil nutrients, and prevent soil erosion (Glover et al., 2010). The decline in soil nutrient availability, particularly nitrogen (N), is an important factor contributing to reduced seed production in intermediate wheatgrass (IWG) stands. Grassland soils typically have low concentrations of available soil nitrogen (Wedin & Tilman, 1990), and as perennial cereal systems mature and resemble grassland ecosystems more closely, nitrogen levels may also decrease. A lack of nitrogen not only limits protein production needed for seed development but has also been linked to reduced seed set (Hacker & Jones, 1971; Hebblethwaite & Ivins, 1978), a trend observed in aging IWG stands (Jungers et al., 2017). Since seed production is critical for optimizing the productivity of perennial cereal systems, nitrogen fertilization may be necessary to support reproduction in older IWG plants. Intermediate wheatgrass (IWG) is not only capable of absorbing significant amounts of nitrogen, but it also demonstrates high nitrogen use efficiency (Jungers et al., 2019; Fagnant et al., 2023). The nitrogen fertilizer requirements for optimizing IWG grain yields are currently less than half the amounts typically applied to maize in the Upper Midwest (Jungers et al., 2019). This efficient nitrogen use is likely due to IWG's extensive fibrous root system, a characteristic shared with other perennial grass cropping systems (Sprunger et al., 2018; Jungers et al., 2015). In fact, IWG has 12–16 times the nitrogen content in its roots compared to annual wheat (Sainju et al., 2017), with estimates ranging from 2 to over 3.5 Mg ha−1 in 2-year-old stands (Pugliese et al., 2019; Bergquist et al., 2022). This combination of grain and forage production potential, coupled with its ability to reduce nitrate leaching, positions

IWG as a promising perennial crop that could contribute to improving the environmental quality of agricultural lands (Dobbratz et al., 2023). The current best practice for nitrogen fertilization in intermediate wheatgrass (IWG) recommends approximately 89 kg ha−1 per year as a single spring application (Jungers et al., 2017). However, second-year IWG stands are often nitrogen-limited, even after the first growing season (Sprunger et al., 2018; Jungers et al., 2017; Reilly et al., 2022). Increasing nitrogen deficiency with stand age has also been identified as a factor contributing to the decline in grain yields observed 2–3 years after establishment (Tautges et al., 2018), which may limit the crop's economic viability. The amount of nitrogen removed by grain and vegetative biomass harvested each year has been estimated at 40–75 kg ha−1 (Kering et al., 2012). While the recommended nitrogen application rate exceeds this removal estimate, additional nitrogen is required to sustain the 2 to 3.5 Mg ha−1 of root biomass, part of which dies and regrows annually. There is still uncertainty about the optimal timing and amount of nitrogen needed to support different plant tissues throughout the growing season. For example, excessive nitrogen applied in the spring can disproportionately increase stem and leaf growth, which may elevate the risk of lodging—where aboveground biomass falls over before grain reaches maturity (Jungers et al., 2017). A key step in improving IWG management will be to better understand the seasonal nitrogen uptake, allocation, and translocation across plant tissues, particularly as stands age, to refine nitrogen application timing and rates for optimal crop performance (Dobbratz et al., 2023).

1.3.7: Functional properties of intermediate wheatgrass

The starch gelatinization temperature of intermediate wheatgrass (IWG) ranges from 62°C to 68°C, which is slightly higher than that of wheat, which falls between 58°C and 64°C (Becker et al., 1991). Additionally, IWG exhibits lower setback and final viscosities compared to wheat,

suggesting a reduced tendency for starch retrogradation. This characteristic is important for bread shelf life, as a lower setback is associated with slower staling during storage (Bharathi et al., 2022; Marti et al., 2015). According to Rahardjo et al. (2018), IWG samples exhibited lower water absorption values compared to wheat. Water absorption refers to the amount of water required to achieve a 500 BU dough consistency, which is considered the optimal consistency for wheat dough. Several factors, including damaged starch content, fiber composition, and the quality of gluten proteins, influence the flour's water absorption capacity. Strong flours tend to have higher water absorption, longer development times, and greater dough stability (Pagani et al., 2014). The lower water absorption, dough development time, and dough stability observed in IWG flours, as compared to wheat, can be attributed to the relatively lower gluten quality of IWG wholegrain flour (Rahardjo et al., 2018). In their 2015 study, Marti et al. examined the protein aggregation kinetics of intermediate wheatgrass (IWG) flour in comparison to hard wheat flour (HWF) using the GlutoPeak tester, a modern tool designed to assess gluten quality. This instrument measures the aggregation behavior of gluten in wheat flour, coarse grains, or vital gluten (Kaur Chandi and Seetharaman, 2012). The gluten aggregation profiles for HWF, IWG flour, and their blends are shown in Figure 2. During testing, the sample is combined with water (in a 1:1 flour-to-water ratio) and subjected to intense mechanical mixing. This process allows the gluten network to form, which results in an increase in the slurry's consistency, reaching a peak value. Following this, continued mechanical stress breaks down the gluten network, leading to a reduction in consistency. The strength of the gluten is determined by the glutenin content, while the ratio of gliadin to glutenin influences the time required to reach the peak (Melnyk et al., 2012). Notably, IWG was found to be capable of forming a gluten network and generating a peak, which contrasts with earlier studies

that reported IWG's lack of gluten-forming ability (Becker et al., 1991). However, IWG exhibited lower peak torque and a shorter time to peak, indicating weaker protein aggregation properties compared to HWF. These differences could be linked to variations in the protein profile, as the glutenin fraction is primarily responsible for gluten strength (peak torque), while the gliadin-to-glutenin ratio has a smaller effect on the peak time (Melnyk et al., 2012).

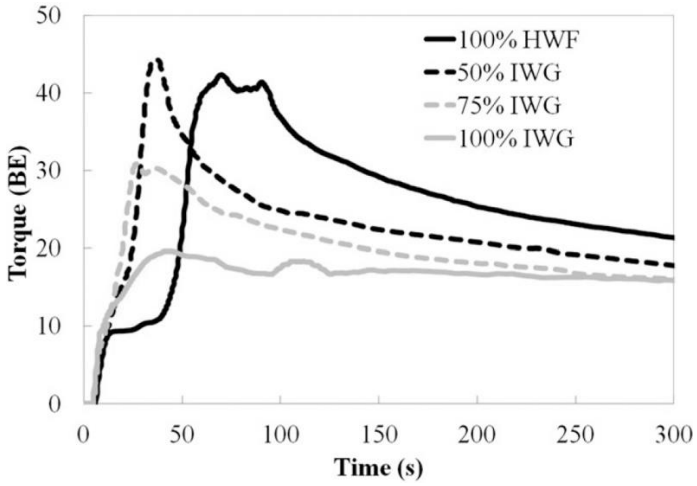

Figure 2:Gluten aggregation properties of hard wheat flour (HWF) and intermediate wheatgrass (IWG) blends measured with a GlutoPeak tester (C. W. Brabender). Sample (8.5 g, 14% moisture) dispersed in 9.5 mL of 0.5M CaCl2 and mixed at 1,900 rpm and 34°C.

1.3.8: Bread baking

The process of baking high-quality bread involves several key steps: mixing, fermentation (proofing), kneading, shaping, and baking. The first stage, mixing, involves combining the liquid and solid ingredients to ensure an even distribution, forming a cohesive mass. During mixing, starch, fiber, and proteins begin to hydrate and interact in an unstructured manner (Rahardjo et al., 2028). With sufficient water, glutenin and gliadin proteins start to bond and form gluten networks. As mixing continues, the dough becomes smoother as the gluten structure develops (DiMuzio, 2009). The farinograph is a tool used to measure the optimal water absorption and mixing time for

dough, with a target consistency of 500 BU for wheat dough (Wheat and Flour Testing Methods, 2004; El-Dash, 1978). Care must be taken not to overmix, as excessive mixing can damage the gluten network, leading to imbalanced dough extensibility and elasticity (DiMuzio, 2009). After mixing, fermentation begins, which is the process of breaking down organic matter (such as carbohydrates) by yeast, bacteria, and molds (DiMuzio, 2009). In bread baking, yeast serves as the fermenting agent, converting sugars into carbon dioxide and alcohol. This process generates gas, causing the dough to rise. The fermentation phase is also referred to as "proofing," and it occurs in three stages: bulk fermentation, intermediate proofing, and final proofing. Bulk fermentation happens immediately after mixing and before dividing the dough. Intermediate proofing occurs between dividing and shaping the dough, while final proofing takes place after the dough is shaped and placed into the pan, allowing it to rest before baking. The timing of each fermentation stage varies depending on the type of bread being made (DiMuzio, 2009). Kneading follows fermentation and plays a crucial role in the formation of the gluten network. It causes protein denaturation, unfolding, and structural reorganization that strengthens the gluten. Kneading also helps align gluten proteins, allowing them to form β-sheets through hydrogen bonding, and fosters the creation of hydrophobic interactions, disulfide bonds, and tyrosine linkages, which improves the dough’s ability to trap air. Additionally, kneading assists with gluten hydration, trapping water between the proteins. As the dough bakes, this water turns to steam, contributing further to the bread's rise (Claire, 2014).

During baking, two important non-enzymatic browning reactions—caramelization and the Maillard reaction—occur. Caramelization takes place when heat is applied to sugars, while the Maillard reaction involves heat, proteins, reducing sugars, and water (Purlis & Salvadori, 2007).

As the dough bakes, it undergoes "oven spring," a rapid expansion caused by the production of carbon dioxide due to accelerated fermentation. However, the bread's surface forms a crust in the intense heat of the oven, which limits further expansion. To prevent this, steam is introduced into the oven before baking to delay the formation of the crust. Too much steam, however, can interfere with the caramelization process, so it is applied just before baking and not during. As baking continues, starch gelatinization and protein coagulation occur, contributing to the final structure of the bread. Water evaporation helps the dough rise further and continues until the bread cools completely. Proper steam release and cooling are essential before cutting or packaging the bread to ensure the best texture and quality (DiMuzio, 2009; Rahardjo et al., 2018).

1.3.9: Bread properties of intermediate wheatgrass

Rahardjo et al. (2018) compared the specific loaf volume of Intermediate Wheatgrass (IWG) with that of wheat and found that IWG loaves generally had lower specific volume and height compared to wheat-based controls. Despite having a higher protein content than wheat, IWG lacks enough High Molecular Weight Glutenin (HMWG) (Bharathi et al., 2022), which likely contributes to the denser texture and reduced specific volume of IWG bread compared to wheat bread (Rahardjo et al., 2018). The increased density of IWG bread was associated with a significant increase in firmness relative to wheat bread (Banjade et al., 2019). The firmer texture of IWG bread is primarily due to its incomplete gluten-forming proteins, which impact the dough's extensibility and elasticity (Cetiner et al. 2023).

In a study by Cetiner et al. (2023), when IWG flour was mixed with wheat flour in varying proportions, it was observed that increasing the amount of IWG flour beyond 15% resulted in firmer bread. This increased firmness was accompanied by a reduction in loaf volume and an

increase in density. A similar trend was observed when comparing loaves made with 100% IWG flour to those made with 100% wheat flour, with IWG loaves being denser and having lower volume (Ferguson et al., 2024).

Additionally, IWG bread tends to expand sideways rather than upwards, which could be due to the higher gliadin-to-glutenin ratio found in IWG compared to wheat. Gliadins contribute to viscosity and extensibility, while glutenins are responsible for elasticity and dough strength. As a result, IWG dough is more viscous than elastic, which may explain the observed sideways expansion during baking (Rahardjo et al., 2018). Furthermore, the higher dietary fiber content of IWG, particularly its insoluble fiber, may also negatively impact the loaf volume (Rahardjo et al., 2018). While IWG is currently used in the food service industry, it still faces several challenges and limitations. Its usage in food production remains minimal in comparison to wheat. Our goal is to increase the adoption of IWG in food applications and broaden the range of products it can be incorporated into (Rahardjo, 2017). This research plays a crucial role in advancing the use of IWG as a food ingredient, particularly through the strategic application of nitrogen fertilizer. Expanding its food applications is a key objective shared by both breeders and food scientists, as they work towards establishing a viable market for IWG as a sustainable food source.

2: MATERIALS AND METHODS

2.1: Sample preparation

Cleaned and dehulled IWG (Variety: TCL cycle 2) samples harvested in 2023 from Rosemount, Minnesota and Madison, Wisconsin were used for this study. For the treated samples, nitrogen (80 lbs./acre) in the form of urea was applied. Wholegrain samples were milled using UDY Cyclone sample Mill equipped with 0.5mm screen (UDY, Fort Collins, CO 80524 U.S.A). Refined IWG

samples were prepared by tempering grains to a moisture content of 14% at 30oC for 4 hours (Bharathi et al, 2022) and milled using the Quadrumat® Junior mill equipped with 0.25mm screen (Brabender GmbH & CO KG – 47055 Duisburg, Germany).

2.2: Nitrogen fertilizer treatment

Intermediate wheatgrass was treated with nitrogen fertilizer in the form of urea, 80 lbs./acre, in the Fall and Spring seasons as reported by Junger et al., 2017 and untreated IWG serving as the control.

2.3: Chemical composition

Moisture content of IWG flour samples was determined in duplicates using Quincy Labs oven (Model 40GC Lab Oven, Hogentogler & Co. Inc. Columbia, MD 21045) at 130°C for 1 hour. (AACC 44-15 A). Protein was determined in duplicates by using the Dumas nitrogen combustion method (AACCI 46-30.01) using a nitrogen analyzer (LECO TruSpecNTM, St. Joseph, MI, USA) with a conversion factor of 5.70. Fat content was determined in duplicates by the Soxhlet method (AOAC 948.22 [12]). Ash content was determined in duplicates by dry ashing method (AACCI 08–01.01). Total carbohydrate was determined by difference. Total, insoluble and soluble dietary fiber were determined following the AACC method 32-07.01. Total starch was determined following the AACCI method 76-13.01.

2.4: Starch pasting properties

Starch pasting properties of flour samples was determined in duplicates using the Micro Visco-Amylo-Graph (MVAG) (Brabender GmbH & Co KG, Duisburg, Germany) (AACCI 22.10), Parameters evaluated were pasting temperature (°C), maximum viscosity (BU), breakdown (BU), and setback (BU).

2.5: Dough mixing rheology

The dough mixing properties of IWG flour samples were evaluated in duplicates using a Brabender Farinograph (C. W. Brabender) equipped with a 10 g bowl according to the AACCI standard method 54-21. Parameters evaluated were water absorption (%), dough development time (s), dough stability (s), and mixing tolerance index (s).

2.6: Protein aggregation kinetics

Protein aggregation kinetics of IWG flour samples were evaluated in duplicate using a Brabender GlutoPeak (C. W. Brabender) based on the method reported by Chandi and Seetharaman (2012), where the peak maximum time (s), maximum torque (BU), and aggregation energy (GPE) were evaluated.

2.7: Physical properties

For thousand kernel weight (TKW), thousand kernels were counted manually in duplicates for samples, and subsequently weighed using the Sartorius weighing balance (Sartorius, practum 224 – IS, 28906164, Sartorius Weighing Technology GmbH, Germany). The length and width of IWG kernel was measured using a digital Vernier caliper (Altraco Inc., Sausalito, California, USA) with calibration to 0.01 mm.

2.8: Amino acid profile

Amino acid analysis was performed, in duplicate, using a method previously described by Kazir et al., 2019 with some modifications.1ml of 6 M HCL was added to about 4 mg of IWG flour samples and hydrolyzed under vacuum at 110^{o}C for 16 hours. Hydrolyzed samples were allowed to cool to room temperature and 100 µl of solution pipetted into an Eppendorf tube. 100 µl of 6 M NaOH was added to neutralize the sample solution. 50 µl was pipetted into an Eppendorf tube and diluted to 2 ml by adding norleucine reagent (4 mmol of norleucine in 1 L of 0.1 M HCL with 20

mg NaN3/L. 1.5 ml of the diluted sample was filtered through nylon syringe filter (0.45 μm) into a high performance HPLC vials. Amino acids were analyzed on a high-performance anion-exchange chromatography (HPAEC) system (Dionex ICS-5000+ HPAEC system, Dionex Corporation) equipped with a pulse amperometric detector (PAD). Separation of amino acids was performed on an Aminopac PA 10 analytical column (2 x 250 mm) protected by its corresponding guard column (2 x 50 mm). Eluents, eluent gradient program, and conditions used were as outlined in application note 163 "Determination of Protein Concentration Using AAA- DirectTM" (Dionex Corporation, 2004) and by Kazir et al. (2019). External standard curves (Linearity range: 0.006-0.025 μM; R2 > 0.98) were constructed using commercial amino acids standard mix AAS18 (Millipore Sigma) for quantification. Data were reported as percentage of specific amino acids to total amino acids.

2.9: Protein secondary structure

IWG dough was prepared using the Brabender Farinograph (C. W. Brabender) equipped with a 50 g according to AACCI standard method 54-21.01, and sampled at the dough development time, as assessed by pretrials. Spectra of the dough were then recorded at least in triplicate on a Bruker Tensor 37ATRFTIR spectrophotometer (Bruker Optics, Inc., Billerica, MA, USA) equipped with a horizontal multireflectance zinc selenide crystal accessory as described by Marti et al., 2016 using OPUS 7.0 software. Protein secondary structures were calculated using second-derivative spectra of amide I regions (1600–1700 cm−1), assigning 1620–1644 cm−1 as β-sheets, 1644–1652 cm−1 as random structures, 1652–1660 cm−1 as α-helix, and 1660–1685 cm−1 as β-turns.

2.10: Dough preparation

Dough for baking bread, AACCI 10–10.03, was used with slight modifications (AACCI Approved Methods, 2010). A mixture of 6.36 g of yeast, 1.8 g of sugar, and 40 g of water was prepared and incubated at 30°C with 85% relative humidity for 20 minutes to activate the yeast. Dry ingredients from Table 1 (except yeast and sugar) were weighed and mixed by hand with a spoon. Activated yeast solution, dry ingredients and the rest of the water were then mixed using a Kitchen aid mixer with a dough hook (Kitchen Aid - KSM 900, Benton Harbor, MI, USA) by mixing at speed 2 for 2 min and speed 4 for 2 min, with scraping down the sides of the bowl after the first mixing. A final moisture of 46.5 g/100 g was targeted.

Table 2:Ingredients and their respective amounts in dough

Ingredients	Amount (g)
IWG flour	120
Yeast	6.36
Salt	1.8
Sugar	1.8
Shortening	3.6
Water	46.5% target moisture

2.11: Bread making

Dough prepared from IWG was spilt into two parts of 102.5 g and placed into the proofer (Baxter PW2E, Orting, WA, USA) at 30 °C and a relative humidity (RH) of 85 for 52 min. After the first proofing, dough samples were hand-punched 10 times and underwent a second proofing for 25 min at 30 °C and RH of 85. After the second proofing, dough samples were punched again 10 times

and proofed for 13 min under the same conditions as above. The dough was then sheeted to 8.6 mm thickness 5 times using an automated sheeter (Sheeter-moulder, National Manufacturing, Division of TMCO Lnc., Lincoln, NE, USA), hand-rolled to a ball, re-sheeted 5 times with the same adjustment, and then rolled to 106 mm length to fit into a trapezoidal Chicago metallic bread pan of size 150 × 83 mm top, 132 × 67 mm bottom, and 57 mm depth (Chicago Metallic, Humboldt, TN 38343 USA) that was sprayed with pan release oil (PAM, Conagra, IL, USA). Pans were placed in the proofer (30 °C and RH 85) for 33 min, then put into a preheated oven and baked at 218 °C for 14 min with steam within the first 10 s (Baxter OV500E1, Orting, WA, USA). Bread removed from the oven was cooled for 1 h, then sliced to 12.5 mm thickness using an automatic bread slicer (Oliver Products Company, Grand Rapids, MI, USA).

2.12: Bread weight and dimensions

Cooled breads (prior to slicing) were weighed with an electronic balance (Scout Pro SP602; Ohaus, Parsippany, NJ, USA). Length, width, and height were measured using a Neiko 01407A digital caliper (Neiko, Taiwan, China). Average length and width were measured at three different positions from the top of the loaf. Average height was measured vertically at three different points of the loaf.

Specific loaf volume was measured using the AACCI rapeseed displacement method 10–05.01 with slight modifications. A bowl was overfilled with rapeseed and levelled with a straight ruler. Then, the same bowl was filled half with rapeseed, the bread was placed and then the bowl was again filled with rapeseed and levelled similarly like before. The rapeseed displaced by the bread were collected and their volume was measured with a graduated cylinder. The specific loaf volume was determined by dividing the loaf volume by its weight (mL/g).

2.13: Bread crumb firmness analysis

Bread crumb firmness was measured using a TA. XT-Plus Texture Analyzer (Texture Technologies) following AACCI method 74–09.01 with TA-5 attachment, a cylindrical probe of 1.3 cm diameter and 3.5 cm length. Four replicates of each slice were analyzed. The force (N) by which the probe deformed the bread was measured using Texture Exponent 32 version 6.0.6.0 software.

2.14: Bread cell count and average cell size

Bread cell counts, average cell size and total cell area in crumb (n = 4) were analyzed with ImageJ version 1.50i (National Institute of Health, Rockville, MD, USA) after converting scanned images with Otsu's auto-threshold, based on a previous study by Banjade et al., 2019.

2.15: Bread crumb color

Crumb color was determined by Chroma Meter CR-221 (Minolta Camera Co., Osaka, Japan) (AACCI method 14.22.01).

2.16: Statistical analysis

One-way analysis of variance (ANOVA) was done using Statgraphics centurion XV to evaluate interactions between nitrogen treatment, refinement, and location. A multiple range test was done to determine significant ($p < 0.05$) difference among the mean values.

3: RESULTS AND DISCUSSIONS

3.1: Chemical composition

The chemical composition of nitrogen treated and untreated intermediate wheatgrass is shown in Table 3. Nitrogen application of IWG significantly ($p < 0.05$) increased the protein content of

refined and whole grain samples from Minnesota and Wisconsin both in fall and spring seasons (Table 3). Adequate nitrogen fertilization promotes the synthesis and accumulation of proteins in grains, thereby changing their physicochemical properties (Wan et al., 2023). The increase in protein content with nitrogen fertilization was more pronounced during the spring than in the fall season. This result indicates that the effect of nitrogen fertilization also depends on the timing of application. Even though nitrogen fertilization can increase protein content in cereal grains, excessive application can lead to a decline in protein content and negatively affect grain quality (Lan et al., 2021). Excessive nitrogen fertilizer application can reduce grain nitrate reductase and glutamine synthetase activities, which are considered crucial enzymes for protein synthesis and accumulation (Wan et al., 2023). This result suggests that the application of 80 lbs./acre of nitrogen fertilizer was sufficient to maintain the high activity of nitrate reductase and glutamine synthetase during protein synthesis and accumulation in IWG. There were some differences in protein content between IWG samples obtained from Minnesota and Wisconsin. This may be attributed to other environmental factors such as precipitation, pH, temperature, and soil type which can affect soil nitrogen availability (Masclaux-Daubresse et al., 2010). Nitrogen treatment of IWG significantly ($p < 0.05$) increased the fat content in both refined and whole grain samples in the fall and spring seasons at both locations. The fat content in all the whole grain samples was higher than that of the refined samples. Fat content in grains is mostly found in the germ which is lost during refining (Šramkováa et al., 2009). There was a significant ($p < 0.05$) decrease in ash content with nitrogen treatment for refined samples from Wisconsin and whole grain samples from Wisconsin and Minnesota in both the fall and spring seasons. Refined samples from Minnesota, however, did not show any significant ($p < 0.05$) difference. Different cereal grains respond differently to nitrogen

fertilizer application in ash content, with some showing decreased ash content. The effect of nitrogen treatment on ash content can vary depending on factors like soil type, climate, and specific agronomic practices. Lewandowski and Kauter (2003) reported that in grain of rye and triticale, the ash content decreased slightly with nitrogen treatment. The ash content of the whole grain samples was higher than that of the refined samples from both locations. The whole grain samples are made from the entire grain of IWG seeds, including the bran, which contains higher amount of minerals than the refined samples (Šramková et al., 2009). The process of grain filling involves the transport of carbohydrates to the kernel and subsequent accumulation of starch (Krishnan & Dayanandan, 2003). Nitrogen promotes the synthesis of carbohydrates and their transport to the grain by promoting photosynthesis. Thus, the effect of nitrogen on carbohydrate transport can vary (Liu et al., 2020). However, Lan et al. (2021) showed that the amount of carbohydrates transported to the grain decreased under high nitrogen conditions, owing to the large amount of carbohydrates consumed for the vigorous growth of vegetative organs such as stems and leaves. Therefore, under high nitrogen and high plant density, less carbohydrate accumulates in the grain (Sun et al., 2019). Owing to the reduction in the amount of substrates caused by the decrease in carbohydrate content in the grain, the synthesis of starch is inhibited, and the grain weight decreases (Jiang, D et al., 2008). The decrease in total starch content was not significant with nitrogen fertilizer application of IWG

Table 3:Chemical composition of nitrogen treated and untreated IWG.

Refinement	Location	Nitrogen treatment	Protein (%)	Fat (%)	Ash (%)	Total starch (%)	Total dietary fiber (%)	Insoluble dietary fiber (%)	Soluble dietary fiber (%)	Total carbohydrate (%)
Refined IWG samples	MN	No treatment	20.51^{a}	2.32^{a}	0.62$^{a\boldsymbol{A}}$	63.20^{a}	6.32^{a}	3.30^{a}	3.02^{a}	76.55$^{C\boldsymbol{B}}$
		Spring treatment	21.91$^{c\underline{a}}$	2.37$^{b\underline{a}}$	0.58$^{a\underline{b}}$	61.95^{a}	6.47^{a}	3.45$^{a\underline{b}}$	3.02^{a}	75.15$^{a\underline{b}}$
		Fall treatment	21.12^{b}	2.34$^{ab\underline{A}}$	0.55^{a}	63.14^{a}	8.33^{b}	5.00^{b}	3.33^{a}	76.04^{b}
	WI	No treatment	21.05^{A}	2.55^{A}	0.79$^{B\boldsymbol{B}}$	64.23^{A}	5.15^{A}	2.70^{A}	2.45^{A}	75.66$^{B\boldsymbol{A}}$
		Spring treatment	2.88$^{B\underline{b}}$	3.03$^{B\underline{b}}$	0.52$^{A\underline{a}}$	64.33^{A}	5.22^{A}	2.28$^{A\underline{a}}$	2.94^{A}	73.58$^{A\underline{a}}$
		Fall treatment	21.10^{A}	2.90$^{AB\underline{B}}$	0.57^{A}	61.32^{A}	6.55^{A}	3.43^{A}	3.12^{A}	75.43^{B}
Wholegrain IWG samples	MN	No treatment	20.53$^{\underline{a}}$	3.62$^{\underline{a}}$	2.41$^{\underline{b}}$	46.39$^{\underline{ab}}$	14.75$^{\underline{a}}$	11.92$^{\underline{a}}$	2.84$^{\underline{a}}$	73.44$^{\underline{c}}$
		Spring treatment	22.83$^{\underline{c}b}$	4.35$^{\underline{b}}$	2.30$^{\underline{a}}$	44.90$^{\underline{a}}$	19.36$^{\underline{b}}$	14.76$^{\underline{a}}$	4.60$^{\underline{b}}$	70.57$^{\underline{a}a}$
		Fall treatment	21.23$^{\underline{a}}$	3.68$^{\underline{a}}$	2.36$^{\underline{ab}}$	47.25$^{\underline{b}}$	17.11$^{\underline{ab}}$	13.35$^{\underline{a}}$	3.76$^{\underline{ab}}$	72.50$^{\underline{b}}$
	WI	No treatment	21.00$^{\underline{A}}$	3.25$^{\underline{A}}$	2.55$^{\underline{B}}$	45.41$^{\underline{A}}$	15.52$^{\underline{A}}$	13.06$^{\underline{A}}$	2.46$^{\underline{A}}$	73.20$^{\underline{B}}$
		Spring treatment	21.70$^{\underline{B}a}$	4.08$^{\underline{B}}$	2.33$^{\underline{A}}$	45.93$^{\underline{A}}$	19.23$^{\underline{B}}$	14.24$^{\underline{A}}$	5.00$^{\underline{B}}$	72.17$^{\underline{A}b}$
		Fall treatment	21.85$^{\underline{B}}$	3.67$^{\underline{AB}}$	2.37$^{\underline{A}}$	45.73$^{\underline{A}}$	17.95$^{\underline{B}}$	14.24$^{\underline{A}}$	3.72$^{\underline{AB}}$	72.11$^{\underline{A}}$

Key, MN – Minnesota, WI – Wisconsin. Mean values with superscript lowercase, uppercase, underline lowercase and underline uppercase letters in each column indicate significant difference ($p < 0.05$) for nitrogen treatment in refined samples from Minnesota, refined samples from Wisconsin, whole grain samples from Minnesota, and Whole grain samples from Wisconsin respectively. Mean values with superscript bolded italic uppercase, italic underline lowercase, italic underline uppercase, bolded italic lowercase, italic lowercase and italic uppercase in each column indicate significant differences ($p < 0.05$) in location for control refined, Spring treated refined, Fall treated refined, whole grain control, Spring treated whole grain and Fall treated whole grain samples respectively.

samples, except for whole grain samples from Minnesota. Total starch content was lower in the whole grain samples than in refined samples from both locations (Table 2). Starch is mostly found in the endosperm and therefore, the lower starch content in the whole grain samples may be attributed to dilution from the bran, which was absent from the refined samples (Šramková et al., 2009). Similarly, nitrogen treatment significantly decreased the total carbohydrate content of IWG in the Fall and Spring seasons of both refined and whole grain samples from both locations., This may be attributed to the decrease in carbohydrate transport to the grain under high nitrogen conditions, owing to the promotion of the growth of vegetative organs, such as stem and leaves, which consume many of the carbohydrates, and may reduce their transport to the grain (Sun et al., 2019). Total carbohydrate content was lower in the whole grain samples than in refined samples from both locations (Table 2). This may again be attributed to dilution from the bran as most of the carbohydrate is concentrated in the endosperm, which was removed during refinement (Šramková et al., 2009). Moreover, nitrogen treatment increased the total dietary fiber content of IWG for both refined and whole grain samples in the Fall and Spring seasons at both locations, some of which were significant ($p < 0.05$) (Table 2). The total dietary fiber content of the whole grain samples was higher than that of the refined samples. This can be attributed to the bran in the whole grain samples, since most of the dietary fiber is in the bran. The high dietary fiber in whole grain may impact on the functional properties and bread baking qualities of IWG (Rahardjo et al., 2018). Similarly, there was an increase in the insoluble dietary fiber content of IWG samples with nitrogen treatment (Table 2). The insoluble dietary fiber content of the whole grain samples was higher than that of the refined samples, and this may be attributed to the bran in the whole grain which is absent in the refined samples due to its removal during refinement. The whole grain IWG,

like most cereals, is richer in dietary fiber (particularly insoluble dietary fiber) than refined IWG (Bharathi et al., 2021). The high insoluble dietary fiber content of the whole grain samples may potentially affect the water absorption capacity and gluten network formation (Wang et al., 2002). Nitrogen treatment did not have any significant effects on the soluble dietary fiber content of the refined samples from both locations. However, the soluble dietary content of the whole grain samples showed a significant ($p < 0.05$) increase with nitrogen fertilizer treatment in the Fall and Spring seasons at both locations. Soluble dietary fiber, like the insoluble dietary fiber, is concentrated in the outer layers of cereal grains (notably the pericarp and aleurone layer) where it may account for up to half of the dry weight but low (often less than 5% dry weight) in the starchy endosperm (Shewry et al., 2023). This may account for the higher soluble dietary fiber content in the whole grain samples than the refined samples, in which most of the pericarp and aleurone layer is lost during refinement. The effect of nitrogen fertilizer application on chemical composition of IWG was more pronounced in the Spring season than in the Fall season. Welch et al. (1966) reported that Spring application of nitrogen is generally more effective than Fall application for winter wheat. This may be ascribed to the fact that Fall application is more susceptible to nitrogen loss through leaching and denitrification. High precipitation during the Fall season favors loss of nitrogen by leaching and denitrification making it less efficient than the Spring application (Welch et al., 1966).

3.2: Starch pasting properties

The starch pasting properties of nitrogen treated and untreated IWG are shown in table 4. The pasting temperatures of IWG flour samples increased with nitrogen treatment, with that of the refined samples from Minnesota and whole grain samples from Wisconsin treated in the Fall and

Spring seasons being significant ($p < 0.05$) (Table 3). The pasting temperature, which represents the temperature at the onset of gelatinization, is strongly related to the ability of starch granules to imbibe water. Thus, factors that affect the ability of starch granules to absorb water, such as the ratio of amylose to amylopectin, the concentration of starch, and the presence of lipids and other components do affect the pasting temperature and other pasting properties of starches (Sasaki et al., 2000). As previously discussed, nitrogen fertilizer treatment increased the protein and fat content of IWG grains (Table 3). The higher pasting temperatures in refined flour samples from Minnesota and the wholegrain samples from Wisconsin may be attributed to the higher fat content which may form starch–lipid complexes. More energy is therefore required to uncoil these starch–lipid complexes resulting in higher pasting temperatures (Kaur & Singh, 2000). The pasting temperatures of the wholegrain IWG flour samples were higher than the refined flour samples regardless of the location where IWG grains were harvested and timing of treatment. The fat content in the wholegrain samples was significantly ($p < 0.05$) higher than that of the refined samples. Fat content in grain is mostly found in the germ and bran which is included in the whole grain but lost during refinement (Šramková et al., 2009). The higher pasting temperatures in the wholegrain samples may be attributed to the higher fat content which may form starch–lipid complexes which may result in higher pasting temperature. Nitrogen treatment significantly ($p < 0.05$) decreased the peak viscosity of IWG in both the whole grain and refined flour samples in the fall and spring seasons irrespective of growing location, except for refined samples from Minnesota which were not significant (Table 4). This observation could be attributed to the presence of more protein and less starch content in the nitrogen treated IWG samples (Table 3). Lim et al. (1999) reported a similar protein effect, where protein content negatively correlated with

peak viscosity but positively correlated with pasting temperature. Protein has intact disulfide bonds that can form a matrix around and/or within the starch granules, making it harder to break the starch granules (Hamaker & Griffin, 1993). Thus, the molecular structure of the protein can prevent swelling, decrease viscosity, and increase pasting temperature (Hamaker & Griffin, 1993). Secondly, nitrogen treated IWG samples have less starch than untreated samples, and therefore the treated samples will be expected to have lower peak viscosity. Gao et al. (2021) also reported that, the peak viscosity of common buckwheat starch was significantly reduced after the application of nitrogen fertilizer, indicating that nitrogen fertilizer can reduce the swelling rate of common buckwheat starch granules, causing the starch granules to absorb water slowly, thereby reducing the starch viscosity.

Table 4:Starch Pasting properties of Nitrogen treated and untreated Intermediate wheatgrass

Refinement	Location	Nitrogen treatment	Pasting temperature (°C)	Maximum viscosity (BU)	Breakdown (BU)	Setback (BU)
Refined IWG samples	MN	No treatment	55.75^{a}	$335.5^{a\boldsymbol{A}}$	182.5^{a}	$410.5^{b\boldsymbol{A}}$
		Spring treatment	56.30^{ab}	$246^{a\underline{a}}$	121^{a}	$268^{a\underline{a}}$
		Fall treatment	56.60^{b}	$296.5^{a\underline{A}}$	143^{a}	$336^{ab\underline{A}}$
	WI	No treatment	56.75^{A}	$515^{B\boldsymbol{B}}$	122.5^{A}	639.5^{AB}
		Spring treatment	56.90^{A}	$510^{B\underline{b}}$	122^{A}	$649^{A\underline{b}}$
		Fall treatment	56.50^{A}	$446^{A\underline{B}}$	117.5^{A}	$578.5^{A\underline{B}}$
Wholegrain IWG samples	MN	No treatment	$58.70^{\underline{a}\boldsymbol{b}}$	$257^{\underline{b}\boldsymbol{a}}$	$109^{\underline{b}}$	$272^{\underline{b}\boldsymbol{a}}$
		Spring treatment	$58.80^{\underline{a}}$	$190.5^{\underline{a}a}$	$83^{\underline{a}}$	$176^{\underline{a}a}$
		Fall treatment	$58.75^{\underline{a}}$	$198.5^{\underline{a}A}$	$84.5^{\underline{a}}$	$190^{\underline{a}A}$
	WI	No treatment	$54.70^{\underline{A}\boldsymbol{a}}$	$404^{\underline{C}\boldsymbol{b}}$	$108.5^{\underline{B}}$	$450.5^{\underline{A}\boldsymbol{b}}$
		Spring treatment	$58.30^{\underline{B}}$	$364.5^{\underline{B}b}$	$83.0^{\underline{A}}$	$374^{\underline{A}b}$
		Fall treatment	$57.70^{\underline{B}}$	$253.5^{\underline{A}B}$	$78.0^{\underline{AB}}$	$450^{\underline{A}B}$

The peak viscosity of refined samples was higher than that of whole grain samples. This may be attributed to the higher dietary fiber content of the whole grain samples, which are removed during refinement. Dietary fiber competes with the starch granule for water, which is needed for starch granule swelling and gelatinization. This can interfere with starch granule swelling and gelatinization of the starch slurry (Collar et al., 2006; Marti et al., 2015b), decreasing the peak viscosity of the whole grain samples. Breakdown viscosity values of IWG decreased with nitrogen treatment in the Fall and Spring seasons, that of whole grain samples from Wisconsin being significant ($p < 0.05$) (Table 2). The breakdown viscosity values demonstrate the stability of the starch granules and indicate the leaching of the amylose and amylopectin out of the starch granule under heat and thus, the lower the breakdown viscosity value, the more stable the starch granules are (Rahardjo et al., 2018). That is, breakdown viscosity values can reflect the ability of starch granules to resist heating. The lower the breakdown viscosity value, the higher the stability indicating that IWG starches had a higher ability to resist heating under higher nitrogen levels (Zhou et al., 2020; Gao et al., 2021). These results indicate that nitrogen treatment could increase the stability of IWG starches which could inform its food application. Generally, nitrogen fertilizer treatment decreased the setback viscosity values of the IWG in both the refined and whole grain samples in the Fall and Spring seasons at both locations (Table 2), some of which were significant ($p < 0.05$). Setback viscosity is an index that measures the stability of starch paste after cooling, and a high setback viscosity indicates a higher tendency of starch pastes to retrograde (Uarrota et al., 2013). This result indicates that nitrogen treatment of IWG samples decreases the tendency of its starch to retrograde. This is consistent with that reported by Gao et al., 2021, in buckwheat at

higher nitrogen treatment. Higher amylose content will therefore result in products with firmer gels upon retrogradation (Thomas & Atwell, 1999).

3.3: Dough rheological properties

Water absorption is the amount of water needed for optimum dough consistency, that is, at 500 BU on the Farinograph (Rahardjo et al., 2018). Water absorption significantly ($p < 0.05$) increased with nitrogen treatment of IWG in both refined and whole grain samples in the fall and spring seasons from both locations, except for whole grain samples from Minnesota which showed a decrease with nitrogen treatment (Table 5). Water absorption of flour is affected by factors such as fiber content and gluten protein quality. Generally, flour with a high level of gluten protein quality absorbs more water because water is needed for gluten formation (Rahardjo et al., 2018). The increase in water absorption with nitrogen treatment may be attributed to an increase in the gluten protein quality of the IWG samples with nitrogen treatment. Apart from wholegrain samples from Minnesota, all the other samples showed a significant ($p < 0.05$) increase in dough development time (DDT) in the fall and spring seasons at both locations. This is consistent with that reported for wheat treated with nitrogen fertilizer (Liu et al., 2022). Since dough development time is regulated by gluten proteins, nitrogen treatment can affect this process (Trevisan et al., 2022). A higher protein content with the application of nitrogen fertilizer has been reported to prolong the DDT for hard winter wheat (Horvat et al., 2022). The increase in DDT was more significant in the whole grain than in the refined samples, at both locations. This was because the bran in the whole grain flour samples caused the dough to take a longer time for the gluten network to form, hence the higher DDT for whole grain samples than the refined samples (Schmiele et al, 2012). Similarly, nitrogen fertilizer treatment increased the dough stability of both refined and whole grain samples

in the fall and the spring seasons at both locations with that of samples from Wisconsin being significant ($p < 0.05$) (Table 4). This is also consistent with that reported for wheat treated with nitrogen fertilizer (Liu et al., 2021). Dough stability depends on levels of high molecular weight glutenin (HMWG) which is lacking in IWG (Rahardjo et al., 2018).

Table 5:Dough rheological properties of nitrogen treated and untreated Intermediate wheatgrass.

Refinement	Location	Nitrogen treatment	WA (%)	DDT (s)	Dough stability (s)	MTI (s)	AT (s)
Refined IWG samples	MN	No treatment	63.35^{ab}	61.5^{a}	$43.0^{a\boldsymbol{B}}$	$234^{a\boldsymbol{B}}$	46.5^{a}
		Spring treatment	$62.80^{a\underline{a}}$	$82.0^{a\underline{a}}$	$50.5^{a\underline{b}}$	$208^{a\underline{b}}$	$60.5^{a\underline{a}}$
		Fall treatment	$64.05^{b\underline{A}}$	88.0^{a}	$52.0^{a\underline{B}}$	$203^{a\underline{B}}$	$57.0^{a\underline{A}}$
	WI	No treatment	$61.00^{A\boldsymbol{A}}$	61.0^{A}	$29.5^{A\boldsymbol{A}}$	$184.5^{B\boldsymbol{A}}$	48.0^{A}
		Spring treatment	$67.35^{B\underline{b}}$	$106.0^{B\underline{b}}$	$41.0^{B\underline{a}}$	$149^{A\underline{a}}$	$77.0^{C\underline{b}}$
		Fall treatment	$67.10^{B\underline{B}}$	87.0^{B}	$36.5^{AB\underline{A}}$	$133^{A\underline{A}}$	$72.0^{B\underline{B}}$
Wholegrain IWG samples	MN	No treatment	$64.75^{\underline{a}\boldsymbol{b}}$	$114.0^{\underline{a}\boldsymbol{a}}$	$57.5^{\underline{a}\boldsymbol{a}}$	$128.5^{\underline{ab}\boldsymbol{b}}$	$89.0^{\underline{a}\boldsymbol{a}}$
		Spring treatment	$64.25^{\underline{a}}$	$134.5^{\underline{b}a}$	$74.5^{\underline{a}a}$	$122.5^{\underline{a}b}$	$101.0^{\underline{b}a}$
		Fall treatment	$63.60^{\underline{a}}$	$111.0^{\underline{a}A}$	$66.0^{\underline{a}A}$	$134^{\underline{b}B}$	$85.0^{\underline{a}A}$
	WI	No treatment	$61.15^{\underline{A}\boldsymbol{a}}$	$145.0^{\underline{A}\boldsymbol{b}}$	$94.0^{\underline{A}\boldsymbol{b}}$	$60.5^{B\boldsymbol{a}}$	$102.0^{\underline{A}\boldsymbol{b}}$
		Spring treatment	$62.70^{\underline{B}}$	$222.0^{\underline{B}b}$	$118.0^{\underline{B}b}$	$43.5^{\underline{A}a}$	$182.5^{\underline{B}b}$
		Fall treatment	$63.05^{\underline{B}}$	$227.5^{\underline{B}}$	$149.5^{\underline{C}B}$	$58^{\underline{AB}A}$	$63.0^{\underline{B}B}$

WA – Water absorption, DDT – Dough development time, MTI – Mixing tolerance index, AT – Arrival time.

The higher the HMWG the higher the dough stability index. Increased dough stability with nitrogen treatment of IWG may be attributed to an increase in HMWG with nitrogen treatment. Nitrogen treatment decreased the dough mixing tolerance index (MTI) of IWG of both refined and

whole grain samples in the Fall and Spring seasons at both locations, with that of samples from Wisconsin being significant ($p < 0.05$). MTI is an important parameter when accessing dough quality (Mann G. et al., 2008). It reflects how quickly the gluten structure breaks down after optimum dough development. Flour with higher MTI, like IWG, indicates less tolerance and more difficulty during mechanical handling and makeup of the dough (Abera G. et al., 2017). The decrease in MTI may be attributed to the increase in gluten protein quality of IWG treated with nitrogen. Arrival time (AT) significantly ($p < 0.05$) increased with nitrogen treatment of IWG in both the refined and whole grain samples in the Fall and Spring seasons at both locations, except for whole grain samples treated in the Fall at Minnesota and Wisconsin which showed a decrease. AT refers to the time from the first addition of water until the top of the curve to first intercept the 500BU dough consistency line. This is also referred to as hydration time (Ahmed et al., 2013). Boehm et al. (2004) reported that nitrogen fertilizer treatment increased the AT of Spring wheat, which is consistent with what was observed with IWG in this study. Shorter AT is desired to speed up the dough mixing process. The AT of whole grain samples were higher than that of the refined samples. This may be attributed to the high bran content of the whole grain samples which compete for water with gluten proteins which increase the time taken for their hydration.

3.4: Protein aggregation kinetics

The application of nitrogen fertilizer significantly ($p < 0.05$) increased the peak maximum time (PMT) of the IWG samples in the Fall and Spring seasons at both locations apart from refined IWG samples from Minnesota (Table 6). PMT indicates the time for complete gluten network formation and corresponds to the time for maximum torque (Grassi et al., 2021). This shows that nitrogen treatment increased the gluten aggregation time in IWG, that is time taken for complete

gluten network formation. This agrees with farinograph DDT, which is also a parameter that measures the time to achieve gluten formation. Apart from whole grain samples from Wisconsin, maximum torque (MT) increased with nitrogen treatment with refined samples treated in the spring from Minnesota and refined samples treated in the Fall and Spring from Wisconsin being significant. Maximum torque corresponds to the maximum peak occurring as gluten aggregates (Grassi et al., 2021). Higher maximum torque indicates a stronger gluten network. The higher maximum torque with nitrogen treatment may be attributed to formation of a stronger gluten network which is desirable in bread baking. Similarly, the application of nitrogen fertilizer significantly ($p < 0.05$) increased the aggregation energy of IWG of both refined and whole grain samples in the Fall and Spring seasons in Minnesota and Wisconsin. Aggregation energy (AgE) corresponds to the area under the GlutoPeak curve and is an indication of gluten network strength. The positive correlation between MT and AgE with nitrogen application may be ascribed to the increase in gluten protein content in the IWG that were treated with nitrogen.

Table 6: Protein aggregation kinetics of nitrogen treated and untreated Intermediate wheatgrass

Refinement	Location	Nitrogen treatment	Peak max. time (s)	Max. torque (BU)	Aggregation energy (GPE)
Refined IWG samples	MN	No treatment	18^{a}	$38^{a\boldsymbol{B}}$	930.51^{a}
		Spring treatment	22.5^{a}	42^{b}	1077.89^{a}
		Fall treatment	23.5^{a}	37^{a}	975.93^{a}
	WI	No treatment	20.5^{A}	$29^{A\boldsymbol{A}}$	759.00^{A}
		Spring treatment	25.5^{B}	40.5^{B}	1156.40^{C}
		Fall treatment	21^{AB}	37^{B}	945.70^{B}
Wholegrain IWG samples	MN	No treatment	$24^{\underline{ab}\boldsymbol{a}}$	$29.5^{\underline{a}\boldsymbol{b}}$	$805.72^{\underline{ab}\boldsymbol{b}}$
		Spring treatment	$26.5^{\underline{b}a}$	$32.5^{\underline{a}b}$	$909.02^{\underline{b}b}$
		Fall treatment	$21.5^{\underline{a}A}$	$28^{\underline{a}B}$	$738.95^{\underline{a}B}$
	WI	No treatment	$30.5^{\underline{A}\boldsymbol{b}}$	$22^{\underline{A}\boldsymbol{a}}$	$621.08^{\underline{A}\boldsymbol{a}}$
		Spring treatment	$35.5^{\underline{AB}b}$	$20.5^{\underline{A}a}$	$621.00^{\underline{A}a}$
		Fall treatment	$40^{\underline{B}B}$	$21.5^{\underline{A}A}$	$635.00^{\underline{A}A}$

3.5: Kernel physical properties

Thousand kernel weight (TKW) is the weight of thousand sound grains. It is a quality test applied to wheat and other grains to determine its potential milling yield. TKW of cereal grains depends on its size and density. The application of nitrogen significantly ($p < 0.05$) increased the thousand kernel weight and length of IWG grains in the fall and spring seasons at both locations (Table 7). The kernel width was however not significantly affected (Table 7). Different studies have reported different responses of TKW of some cereals to nitrogen application. In a Swedish study, it was reported that TKW of different varieties of oats were not affected by nitrogen application (Valkama et al., 2013). Esala and Larpes (1986) also reported that an experiment conducted in clay soil demonstrated an increase in TKW with nitrogen treatment of wheat (50 – 200 Kg ha-1), but in barley, no increase was observed with nitrogen application (100 Kg ha-1). Raja (2001) also reported a positive response in kernel weight with nitrogen treatment in maize. Increasing nitrogen rates increased the activity of enzymes in maize which increased the kernel weight (Amanullah et al., 2009). The increase in TKW and length of IWG may be attributed to the increase in the activities of enzymes in IWG which increase the TKW and size of IWG kernel.

3.6.: Amino acid profile

Amino acids content is one of the most vital characteristics of the biological value of grain and are the structural units of Protein (Nokerbekova et al., 2018). The amino acid profile of nitrogen treated, and untreated IWG flour samples is as shown in Table 8. The effect of nitrogen fertilization on IWG flour samples varied across the seventeen different amino acids (table 8). Samples treated in the spring generally increased with nitrogen treatment compared to untreated samples regardless

Table 7: Thousand kernel weight, kernel length and width of nitrogen treated and untreated IWG

Location	Nitrogen treatment	Thousand Kernel weight (g)	Length of Kernel (mm)	Width of kernel (mm)
MN	No treatment	7.32^{aB}	5.29^{a}	1.39^{a}
	Spring treatment	$7.45^{b\underline{A}}$	5.78^{b}	1.47^{a}
	Fall treatment	$7.57^{b\underline{a}}$	5.56^{b}	1.47^{a}
WI	No treatment	7.10^{AA}	5.34^{A}	1.44^{A}
	Spring treatment	$7.62^{B\underline{B}}$	5.79^{C}	1.51^{A}
	Fall treatment	7.81^{Ab}	5.52^{B}	1.51^{A}

Mean values with superscript lowercase and uppercase letters in each column indicate significance difference ($P < 0.05$) for nitrogen treatment in IWG samples from Minnesota and Wisconsin respectively. Mean values with superscript italic uppercase, italic underline uppercase and italic underline lowercase letters in each column indicate significant difference ($p < 0.05$) at location in nitrogen treatment for control, Spring treatment and Fall treatment samples respectively.

of growing location. The increase in arginine, serine, leucine, phenylalanine, glutamine, and tyrosine content in spring treated refined samples from MN, and serine content in spring treated samples from WI were significant ($p < 0.05$). For whole grain samples treated in the spring, except for alanine and prolamin, the amino acid content increase was significant ($p < 0.05$) with nitrogen treatment. Compared to the non- treated samples, nitrogen treatment did not have any significant ($p < 0.05$) effect on samples treated in the fall irrespective of growing location and refinement. The application of nitrogen fertilizer enhanced the nutritional quality of the IWG samples that were treated in the spring by increasing the essential amino acid content. These results suggest that nitrogen application in the spring had a greater impact on the amino acid composition of intermediate wheatgrass compared to fall application. Brown and Petrie, 2006 found that spring top dressed nitrogen was the most effective nitrogen timing for protein. This may mean that applying nitrogen in the spring is generally more effective for winter red wheat than fall

application. This is because nitrogen applied in the fall is more prone to loss through leaching and denitrification, especially with high precipitation during the season, making spring application more efficient (Brown & Petrie, 2006).

Table 8:Total amino acids content of nitrogen treated and non-treated intermediate wheat grass

Refinement	Location	Nitrogen treatment	Arg (%)	Lys (%)	Ala (%)	Thr (%)	Gly (%)	Val (%)	Ser (%)	Pro (%)	Iso (%)	Leu (%)	Met (%)	His (%)	Phy (%)	Glu (%)	Asp (%)	Cyt (%)	Tyr (%)
Refined IWG samples	MN	No treatment	2.87^{ab}	0.95	0.08	0.62	0.73	1.15	0.74^{ab}	0.29	0.24	0.76^{a}	1.55	1.33$^{\boldsymbol{B}}$	0.97^{a}	0.85^{a}	0.12	0.51	0.59^{a}
		Spring treatment	3.72^{bb}	1.34	0.16$^{\underline{b}}$	0.88	0.95	1.58	1.09^{b}	0.45$^{\underline{b}}$	0.48$^{\underline{b}}$	1.07$^{b\underline{b}}$	2.10	1.65$^{\underline{b}}$	1.34^{b}	1.21^{b}	0.18	0.66	0.89^{b}
		Fall treatment	2.68^{a}	0.97	0.16	0.58	0.65	1.02	0.60^{a}	0.41	0.37	0.71^{a}	1.42	1.11	0.87^{a}	0.83^{a}	0.10	0.47	0.52^{a}
	WI	No treatment	2.21	0.65	0.03	0.28	0.61	0.51	0.21^{A}	0.12	0.04	0.27	0.62	0.75$^{\boldsymbol{A}}$	0.60	0.60	0.07	0.32	0.28
		Spring treatment	2.49$^{\underline{a}}$	0.84	0.01$^{\underline{a}}$	0.39	0.57	0.75	0.76^{B}	0.13$^{\underline{a}}$	0.09$^{\underline{a}}$	0.41$^{\underline{a}}$	0.85	0.71$^{\underline{a}}$	0.84	0.77	0.15	0.41	0.33
		Fall treatment	2.13	0.65	0.04	0.31	0.37	0.56	0.29^{A}	0.10	0.09	0.31	0.63	0.68	0.61	0.57	0.08	0.30	0.28
Whole grain IWG samples	MN	No treatment	3.62$^{\underline{ab}}$	0.94$^{\underline{a}}$	0.11	0.52$^{\underline{ab}}$	0.51$^{\underline{ab}}$	0.90$^{\underline{ab}}$	0.55$^{\underline{a}\boldsymbol{A}}$	0.30	0.25$^{\underline{ab}}$	0.49$^{\underline{a}}$	1.05$^{\underline{ab}}$	1.02$^{\underline{a}}$	0.68$^{\underline{a}}$	0.64$^{\underline{a}}$	0.11$^{\underline{a}}$	0.32$^{\underline{a}}$	0.43$^{\underline{a}}$
		Spring treatment	5.28$^{\underline{b}}$	1.56$^{\underline{b}}$	0.15	0.80$^{\underline{b}}$	0.85$^{\underline{b}}$	1.37$^{\underline{b}}$	0.94$^{\underline{b}}$	0.48	0.37$^{\underline{b}}$	0.84$^{\underline{b}}$	1.67$^{\underline{b}}$	1.61$^{\underline{b}}$	1.17$^{\underline{b}}$	1.02$^{\underline{b}}$	0.22$^{\underline{b}}$	0.60$^{\underline{b}}$	0.74$^{\underline{b}}$
		Fall treatment	3.30$^{\underline{a}}$	0.91$^{\underline{a}A}$	0.05^{A}	0.43$^{\underline{a}A}$	0.45$^{\underline{a}}$	0.71$^{\underline{a}A}$	0.39$^{\underline{a}A}$	0.16	0.11$^{\underline{a}A}$	0.36$^{\underline{a}A}$	0.73$^{\underline{a}A}$	0.95$^{\underline{a}}$	0.65$^{\underline{a}}$	0.60$^{\underline{a}}$	0.12$^{\underline{a}}$	0.35$^{\underline{a}}$	0.36$^{\underline{a}}$
	WI	No treatment	3.74	1.15	0.12	0.63	0.62	1.09	0.62$^{\boldsymbol{B}}$	0.37	0.33	0.63	1.28	1.05	0.77	0.67	0.16	0.38	0.43
		Spring treatment	3.92	1.18	0.11	0.72	0.71	1.29	0.66	0.35	0.32	0.76	1.50	1.21	0.91	0.78	0.18	0.44	0.57
		Fall treatment	4.05	1.24^{B}	0.12^{B}	0.80^{B}	0.73	1.35^{B}	0.75^{B}	0.35	0.40^{B}	0.84^{B}	1.62^{B}	1.20	0.88	0.80	0.17	0.48	0.59

Key: MN-Minnesota, WI-Wisconsin, Arg-Arginine, Lsy-Lysine, Ala-Alanine, Thr-Threonine, Gly-Glycine, Val-Valine, Ser-Serine, Pro-Prolamin, Iso-Isoleucine, Leu-Leucine, Met-Methionine, His-Histidine, Phy-Phenylalanine, Glu- Glutamine, Asp-Asparagine, Cyt-Cysteine, Tyr-Tyrosine.

Mean values with superscript lowercase, uppercase, underline lowercase and underline uppercase letters in each column indicate significant difference ($p < 0.05$) for nitrogen treatment in refined samples from Minnesota, refined samples from Wisconsin, whole grain samples from Minnesota, and Whole grain samples from Wisconsin respectively. Mean values with superscript bolded italic uppercase, italic underline lowercase, italic underline uppercase, bolded italic lowercase, italic lowercase and italic uppercase in each column indicate significant differences ($p < 0.05$) in location for control refined, Spring treated refined, Fall treated refined, whole grain control, Spring treated whole grain and Fall treated whole grain samples respectively.

3.6.: Protein secondary structure

In all IWG samples, the primary protein secondary structures were β-sheets and β-turns, with random structures and α-helices present in smaller amounts (Table 9). This is consistent with that reported by Banjade et al., 2019. It has been indicated in previous studies that in dough model system (i.e. hydrated gluten) β-turns are the dominant structure, followed by β-sheets (Bock and Damodaran, 2013). This agrees with the IWG dough system in this study. The loop-and-train model suggested by Belton (1999) describes β-turns in gluten as flexible loop structures where proteins interact with water, while β-sheets are seen as train structures where proteins are more closely bonded to each other (Banjade et al., 2019). The fact that IWG dough has fewer β-sheets than β-turns reflects the lack of gluten network strength, possibly due to deficiency in HMW-GS resulting in fewer protein-protein interactions (Tyl and Ismail, 2019). Refined IWG dough samples treated with nitrogen fertilizer had an increase in β-sheets and a decrease in β-turns than non-treated IWG dough regardless of where IWG was harvested. (Table 8). However, in whole grain IWG dough samples, nitrogen treatment increased the β-turns and reduced the β-sheets compared to the non-treated from MN and WI. The exception to this trend is the fall treated whole grain samples from WI which had an increase in β-sheets and a decrease in β-turns than the non-treated IWG samples (Table 8). These results show that nitrogen treatment enhanced the viscoelastic network of refined IWG dough, possibly by enriching the HMW-GS profile of IWG. The presence of bran constituents in whole grain IWG dough may have rearranged the structure of gluten-forming proteins in whole grain IWG dough (Banjade et al., 2019) increasing the β-turns and decreasing the β-sheets. Nitrogen fertilization did not have a significant ($p < 0.05$) impact on Alpha helix and Random structure of IWG dough samples regardless of timing of treatment, refinement,

and growing location, with the exclusion of refined samples from Minnesota which had Alpha helix significantly ($p < 0.05$) increasing with nitrogen treatment. These results suggest that nitrogen treatment enhanced the dough strength of refined samples by increasing β-sheets and decreasing β-turns in refined IWG flour samples. The β-turns and β-sheets of the IWG dough samples varied across the locations where they were harvested.

Table 9:Protein secondary structures in dough made from nitrogen treated and non-treated intermediate wheatgrass.

Refinement	Location	Nitrogen treatment	Beta turns (%)	Alpha helix (%)	Random structures (%)	Beta sheets (%)
Refined	MN	No treatment	42.23^{b}***A***	6.68^{a}***A***	20.60***B***	30.49***B***
		Spring treatment	41.15^{a}*a*	10.52^{b}	17.61	30.72
		Fall treatment	41.35^{a}*A*	10.34^{b}	17.14	31.17*B*
	WI	No treatment	49.27^{B}***B***	9.67***B***	14.81***A***	26.25^{A}***A***
		Spring treatment	43.76^{A}*b*	10.05	14.75	31.44^{B}
		Fall treatment	47.45^{B}*B*	7.90	16.52	28.13^{AB}*A*
Wholegrain	MN	No treatment	33.38^{a}***a***	9.88	19.07	37.67^{b}***b***
		Spring treatment	36.17^{b}*a*	10.34	19.78	33.70^{a}*b*
		Fall treatment	38.91^{c}	11.47	17.56	32.05^{a}
	WI	No treatment	40.52^{AB}***b***	10.16	18.69	30.62^{AB}***a***
		Spring treatment	41.67^{B}*b*	9.63	19.54	29.17^{A}*a*
		Fall treatment	38.36^{A}	11.67	18.12	31.86^{B}

3.7: Bread properties

3.7.1: Loaf height

The loaf height of nitrogen treated and non-treated IWG samples is shown in Table 10. Figure 3 shows slices of bread from nitrogen treated and untreated whole grain and refined IWG flour. The application of nitrogen fertilizer significantly ($p < 0.05$) increased the loaf height of refined samples from MN that were treated in the spring and fall seasons. This may be attributed to better gluten development in these samples due to an increase in HMW-GS (Tyl and Ismail, 2019). Nitrogen fertilization is known to enhance HMW-GS profile in wheat (Rossmann et al., 2020). The bread loaf height of refined IWG samples were higher than wholegrain samples regardless of where it was harvested and the timing of application (Table 10). This may be attributed to the bran content of the wholegrain samples. Bran is known for competing for water with gluten protein which results in the weakening of the gluten protein network thereby reducing the gas holding property of wholegrain bread. Secondly, the bran in whole grain, which is mainly dietary fiber, can interfere with the formation of crosslinks between gluten proteins and between gluten and starch. This disruption weakens the gluten network structure, leading to a less cohesive gluten matrix (Mengyuan et al., 2023).

3.7.2: Loaf specific volume

Nitrogen treatment did not have a significant impact on the loaf specific volume of both refined and wholegrain IWG samples grown in MN and WI (Table 10). Bread specific volume is parameter for baking quality evaluation (Rossmann et al., 2020) and was determined by dividing the loaf volume over the loaf weight. Baking quality depends on the composition of storage proteins and therefore the impacts of nitrogen fertilizer treatment on the amount and proportions of gluten proteins is of a particular interest (Rossmann et al., 2020). Johanson et al., 2001 reported that

nitrogen application affects the gluten strength as well as bread volume. The application of nitrogen fertilizer is known to promote the accumulation of glutenin and gliadin fractions in wheat grains (Zheng et al., 2018). These results indicate that, while nitrogen fertilizer application increases the storage protein content of IWG grains, it might not necessarily constitute an appreciable amount to affect the loaf specific volume of IWG bread irrespective of the growing location. The loaf specific volume for refined IWG samples was more than wholegrain samples. Banjade et al., 2019 reported that bran content negatively correlated with loaf volume, Bran physically disrupts the foam structure of the dough and minimized the retention of gas during fermentation, which results in reduction in bread loaf volume (Majzoobi et al., 2014). The lower loaf specific volume of the wholegrain samples may be attributed to the bran content which was removed from the refined samples during the refining process. The specific volume of IWG samples from MN were significantly higher than samples from WI regardless of the timing of application of nitrogen.

3.7.3: Crust color

The mean L* values for crust color is as shown in table 10. There was no significant difference in crust color with nitrogen treatment, except for whole grain samples from MN. Refined samples from WI were significantly ($p < 0.05$) lighter than those from MN without regard to the timing of nitrogen application.

Table 10:Bread properties of nitrogen treated and non-treated intermediate wheat grass

Refinement	Location	Nitrogen treatment	Height (cm)	Specific Volume (ml/g)	Crust color (L*)	Firmness (Young modulus/knm^2)	Average air cell size	Cell count
Refined IWG samples	MN	No treatment	33.03$^{a\boldsymbol{A}}$	1.97$^{\boldsymbol{B}}$	58.10$^{\boldsymbol{A}}$	33.30$^{a\boldsymbol{A}}$	29.28	56.67
		Spring treatment	35.27^{b}	2.12$^{\underline{b}}$	56.30$^{\underline{a}}$	38.38^{b}	27.70	71.67
		Fall treatment	35.82^{ab}	2.03$^{\underline{B}}$	56.90$^{\underline{A}}$	38.82$^{b\underline{B}}$	27.39	75.00
	WI	No treatment	36.18$^{A\boldsymbol{B}}$	1.55$^{\boldsymbol{A}}$	64.30$^{\boldsymbol{B}}$	35.56$^{B\boldsymbol{B}}$	25.21^{B}	68.67
		Spring treatment	35.20^{A}	1.53$^{\underline{a}}$	62.40$^{\underline{b}}$	38.04^{C}	22.38^{A}	72.33
		Fall treatment	34.47^{A}	1.62$^{\underline{A}}$	63.40$^{\underline{B}}$	32.85$^{A\underline{A}}$	24.02^{AB}	65.17
Wholegrain IWG samples	MN	No treatment	31.72$^{\underline{a}}$	1.33$^{\boldsymbol{b}}$	46.20$^{b\boldsymbol{a}}$	133.20$^{b\boldsymbol{a}}$	31.93$^{\underline{ab}}$	143.33$^{b\boldsymbol{b}}$
		Spring treatment	32.57$^{\underline{a}}$	1.28	46.30$^{b\underline{a}}$	123.52$^{\underline{a}a}$	35.45$^{\underline{b}}$	130.00$^{\underline{ab}b}$
		Fall treatment	33.73$^{\underline{a}}$	1.34^{B}	44.70$^{\underline{a}A}$	121.44$^{\underline{a}A}$	30.04$^{\underline{a}}$	124.67$^{\underline{a}A}$
	WI	No treatment	29.37$^{\underline{A}}$	1.11$^{\boldsymbol{a}}$	47.50$^{\underline{A}\boldsymbol{b}}$	145.26$^{\underline{B}\boldsymbol{b}}$	32.36$^{\underline{AB}}$	114.33$^{\underline{B}\boldsymbol{a}}$
		Spring treatment	28.95$^{\underline{A}}$	1.18	50.10$^{\underline{B}b}$	142.29$^{\underline{B}b}$	43.82$^{\underline{B}}$	57.66$^{\underline{A}a}$
		Fall treatment	29.95$^{\underline{A}}$	1.18^{A}	50.60$^{\underline{B}B}$	134.70$^{\underline{A}B}$	24.02$^{\underline{A}}$	135.00$^{\underline{B}B}$

3.7.4: Bread firmness.

Table 10 shows the results of the effect of nitrogen fertilizer application on the crumb firmness of IWG. Crumb firmness significantly ($p < 0.05$) increased with nitrogen treatment in refined IWG samples in the spring and fall seasons at both locations except for the refined sample from WI that was treated in the fall. However, excluding whole grain samples treated in the spring from WI, breadcrumbs from whole grain IWG flour showed a significant ($p < 0.05$) decrease in firmness with nitrogen fertilizer application in the spring and fall seasons. Studies have shown that gliadins contribute to the viscosity of the viscoelastic gluten, while glutenin's, especially HMW glutenin's, contribute to the elasticity and strength of gluten (Bonilla et al.,2020). IWG is deficient in HMWG, but rich in gliadins (Rahardjo et al., 2018). The higher proportion of gliadins to glutenin's in IWG may have contributed to the increase in crumb firmness of the refined IWG samples with nitrogen fertilization. The decrease in crumb firmness in the whole grain samples with nitrogen treatment may be due to dilution of gliadin rich gluten with bran in the whole grain samples which is absent in the refined samples.

3.7.5: Bread crumb cell structure.

The size of and distribution of air cells, also known as crumb cells, are important factors in determining the quality and acceptability of bread (Angioloni et al., 2009). Factors such as dough composition (gluten quality) (Ragaee et al., 2006), starch pasting characteristics (Ragaee et al., 2006), dough formulation (Brennan et al., 1996), alpha amylase activity of flour (Tohver et al., 2005), affect bread crumb structure. The average crumb cell size and cell count of nitrogen non-treated and treated are shown in Table 10. Nitrogen treatment did not have any significant ($p < 0.05$) impact on crumb cell size of the refined IWG bread samples from MN, it however reduced the crumb cell size of the refined IWG bread samples from WI regardless of the timing of

treatment. Reduced average cell size indicates more and smaller gas cells (Banjade et al., 2019). Average crumb cell size significantly ($p < 0.05$) increased with nitrogen fertilization in whole grain samples treated in the spring and decreased in whole grain samples treated in the fall. Cell counts of refined samples were not significantly ($p < 0.05$) affected by nitrogen fertilizer application irrespective of timing of treatment and growing location. However, nitrogen treatment decreased cell counts in whole grain samples, apart from fall treated samples from WI. A higher count can be viewed as favorable as the number of alveoli represents air entrapped in the crumb (Salinas & Puppo, 2015). These results indicate that nitrogen treatment enhanced the production of more and smaller gas cells in refined samples from WI but negatively impacted on the production of gas cells in whole grain IWG bread samples. Except for Spring treated whole grain samples that showed a bigger average cell size for samples from WI compared to whole grain samples from MN, there were no significant ($p < 0.05$) differences in average cell size between samples harvested in WI and MN. There were no differences in cell counts between breads from refined IWG from WI and those from MN. Cell counts were higher for whole grain samples from MN compared to whole grain samples from WI, except for Fall treated whole grain samples from WI which were higher. Bran is known to affect cell areas of bread baked from IWG flour (Banjade et al., 2019).

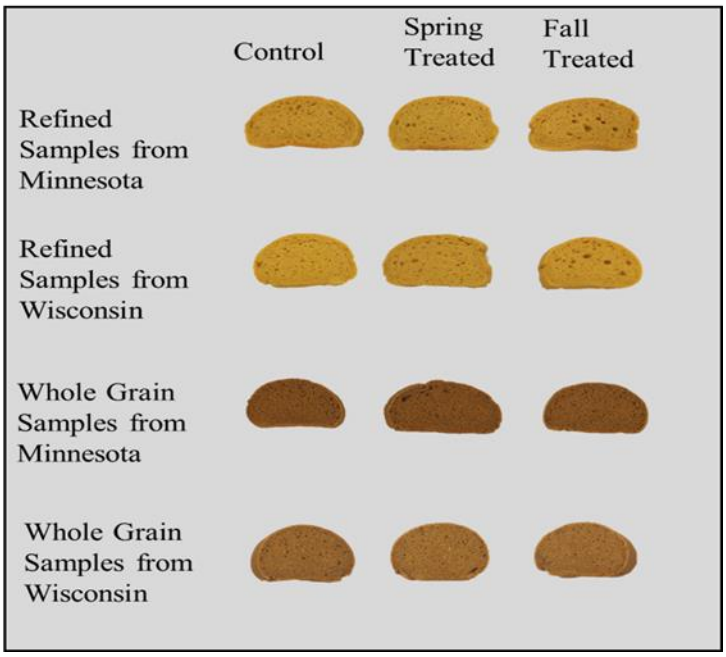

Figure 3:Bread slices of nitrogen treated and non-treated intermediate wheatgrass from Minnesota and Wisconsin.

4.0: CONCLUSION

Nitrogen application had varied effects on the physico-chemical and functional characteristic of IWG. The chemical composition of IWG was impacted in different ways. While protein and fat contents significantly increased in the whole and refined samples, there were no clear trends in the other chemical parameters. The nitrogen effects on the other parameters depended on whether they were refined or not and the location. The weight of the seeds significantly increased particularly in the fall season for both locations. The max torque and energy needed to aggregate IWG protein indicated that nitrogen application resulted in stronger doughs, especially for spring application irrespective of location. This observation was confirmed with an increase in dough development time and stability on the Farinograph. Starch hot paste viscosity generally decreased with nitrogen fertilization, so as starch break down and setback.

The effect of nitrogen fertilizer application varied across the seventeen amino acids depending on time of treatment and growing location. It generally increased in IWG samples treated in the spring while samples treated in the fall were not significantly affected with the application of nitrogen fertilizer. Nitrogen treatment led to an increase in β-sheets and a decrease in β-turns in refined samples, whereas whole grain samples showed the opposite trend, except for Fall-treated samples from Wisconsin. Nitrogen treatment increased loaf height in refined samples from Minnesota but did not affect loaf specific volume. It increased bread firmness in refined samples while reducing firmness in whole grain samples. Cell count decreased in whole grain bread samples with nitrogen treatment, except for Fall-treated samples from Wisconsin, where it increased. These results indicate that nitrogen fertilizer application influences the nutritional profile, protein secondary structures, and bread quality of intermediate wheatgrass in varied ways. This results on the effect of nitrogen on IWG chemical composition and functional properties, and bread properties could inform its food application.

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