Morphological and biochemical properties of Australian native grains

by

Farkhondeh Abedi

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Statement of Originality

This is to certify that, to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Farkhondeh Abedi 25th June 2025

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Authorship Attribution Statement

Chapter 2 of this thesis is an expanded version of the following published journal article (a copy of which is in the Appendix):

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I, **Farkhondeh Abedi** (first author), in discussion with my Lead Supervisor, Thomas H. Roberts, and Auxiliary Supervisors, Claudia Keitel, and Ali Khoddami, designed and executed the experiments, gathered data, processed, and analysed the data; prepared the original draft of all articles. The contributions of authors other than myself are as follows:

Chapter 2. Thomas H. Roberts: Conceptualization; Writing–review and editing; Supervision; Project funding and administration. Claudia Keitel: Conceptualization; Resources; Writing–review and editing; Supervision. Ali Khoddami: Methodology, Writing–review, and editing. Angela Pattison: Resources; Writing–review and editing. Salla Marttila: Methodology; Writing–review and editing.

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In addition to the above statement, as the first author, I obtained copyright permission from the relevant journal publisher to include published material in this thesis.

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Use of Generative AI

No generative AI tools were used in the preparation of this thesis.

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Abbreviations

Abbreviation	**Full Term**
AA	α-amylase
ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
ACN	Acetonitrile
AMG	Amyloglucosidase
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BD	Bulk density
BG	Button Grass
BP	Before Present
CAA	Chloroacetamide
CCNFSDU	Codex Committee on Nutrition and Foods for Special Dietary Uses
DF	Dietary fibre
DFAT	Department of Foreign Affairs and Trade
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organisation of the United Nations
FDR	False discovery rate
FFA	Free fatty acids
GOPOD	Glucose oxidase-peroxidase
HMW	High-molecular-weight
IPCC	Intergovernmental Panel on Climate Change
IRRI	International Rice Research Institute
LC-MS	Liquid chromatography-mass spectrometry
LEA	Late embryogenesis abundant
LFD	Lateral flow device
LMW	Low-molecular-weight
MDB	Murray-Darling Basin
MG	Curly Mitchell Grass
MUFA	Monounsaturated fatty acids

NM	Native Millet
OAC	Oil absorption capacity
PAA	Porcine pancreatic α-amylase
PUFA	Polyunsaturated fatty acids
PVPP	Polyvinylpolypyrrolidone
RS	Resistant starch
RT	Room temperature
RVA	Rapid visco analyser/analysis
SD	Standard deviation
SDC	Sodium deoxycholate
SDC-FASP	Sodium deoxycholate–filter-aided sample preparation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SL	Seed length
SW	Seed width
TCEP	Tris-(2-carboxyethyl)phosphine
TEAB	Triethylammonium bicarbonate
TFA	Trifluoroacetic acid
TTC	2,3,5-triphenyltetrazolium chloride
WAC	Water absorption capacity
WG	Weeping Grass

Abstract

Recognition of the value of native grasses to the sustainability of agro-ecosystems throughout Australia is becoming established, but grain production from native grasses remains a small, niche industry. Expanded production of Australian native grains has the capacity to contribute to health-focused food innovation, ecological resilience, and the revitalisation of First Nations food systems. This expansion must be led by Indigenous communities, whose enduring cultural connection to these grains and landscapes is fundamental. This PhD research involved valuable consultation with Indigenous people and acknowledges their custodianship of Country over millennia.

This thesis aimed to evaluate four Australian native grasses—*Dactyloctenium radulans* (Button Grass), *Astrebla lappacea* (Curly Mitchell Grass), *Panicum decompositum* (Native Millet), and *Microlaena stipoides* (Weeping Grass)—as underutilised species with potential in flour blends with wheat and other domesticated cereals. This was achieved by characterising their grains and whole-grain flours in tersm of microstructural features, proximate composition, carbohydrate and protein profiles, and functional properties, and by interpreting how these traits influence both nutritional value and technological behaviour in food processing.

Microscopy revealed distinctive structural attributes, including thinner endosperm cell walls and larger embryos than wheat, which may enhance nutrient density and digestibility. Weeping Grass possessed a two-cell-thick aleurone and elevated β-glucan content, traits known to influence water-binding capacity, viscosity, and cholesterol-lowering effects. Proximate analysis demonstrated that Curly Mitchell Grass had exceptionally high protein levels (29 g/100 g db), supporting applications in high-protein foods, while Native Millet contained elevated lipid levels (8 g/100 g db), which may enhance energy density and sensory qualities. Functional property analysis showed that hydration, bulk density, and foaming varied among species, reflecting their biochemical profiles; β-glucan was associated with higher water and oil absorption, while amylose contributed to gel strength and foam stability.

Carbohydrate analysis revealed substantial differences in starch quality. Curly Mitchell Grass exhibited the highest amylose content (41.2% of total starch), followed by Native Millet, both exceeding values typically reported for wheat, barley, and sorghum. Button Grass contained the highest dietary fibre (21.9 g/100 g db), while Weeping Grass had β-glucan concentrations comparable to high β-glucan barley varieties. These features suggest potential for use in high-fibre, gluten-free, and functional foods with enhanced textural and health benefits. Protein composition influences nutritional quality, allergenicity, and functionality, making its characterisation critical for evaluating grains traditionally used by Indigenous communities but not commonly incorporated into industrial food systems. Osborne fractionation and SDS-PAGE revealed substantial differences in the distribution of protein solubility fractions and molecular profiles, respectively, across the native grains with barley as the comparison grain. Curly Mitchell Grass and Weeping Grass had higher glutelin content than barley, while Button Grass had the highest proportion of prolamins among the native species. The highest albumin proportion among the native grains was found in Native Millet, which surpassed that of barley.

Proteomic analysis identified abundant stress-associated proteins, including heat shock proteins, which appeared among the 20 most abundant proteins in most native species at higher levels than in barley, likely reflecting adaptation to arid and variable environments. These results provide new insights into the unique protein profiles of native grains and their potential roles in specialised food applications, including gluten-free and those contributing to sustainable and nutritionally resilient diets.

Overall, this research demonstrates that Australian native grains combine unique structural, nutritional, and functional properties that distinguish them from domesticated cereals. By linking microstructure and composition to processing behaviour and health outcomes, this study provides novel scientific insights and establishes a foundation for developing innovative food products that support both consumer health and Indigenous-led enterprises.

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Chapter 1

Introduction

1.1 Crop Production in Australia

1.1.1 Indigenous Australian Agricultural Practices (Pre-European)

Sydney Parkinson, one of the botanical draughtsmen onboard Captain James Cook's ships, explained what he saw when he arrived in Australia for the first time: "The country looked very pleasant and fertile, and the trees, quite free from underwood, appeared like plantations in a gentleman's park." (Fletcher *et al.*, 2021). At that time, no European had any idea of the enormous extent of vegetation management that had been ongoing in Australia for tens of thousands of years by First Nations people. They, in turn, had no idea of the scale of the changes in vegetation across the continent that would be carried out within a few decades of colonisation.

Before European arrival, First Nations people lived in tribal groups, and each group had responsibilities for preserving the landscape. First Nations people are influential on the past, present, and future of Australia's landscape and vegetation. Evidence shows that Indigenous practices, including species relocations, burning, and water management, played a key role in shaping Australian pre-colonial environments. Before European settlement, plant species with edible roots—used as a food source—comprised 10% of the flora in grasslands and woodlands in southeastern Australia. Digging various species, including yam-daisies (*Microseris* species) for their edible tubers, corms, and bulbs, improved the soil nutritionally by aerating and loosening the soil (Ens *et al.*, 2017; Gott, 2008; Gott *et al.*, 2015). Indigenous people facilitated the vegetative reproduction of tubers and made softer, aerated soil beds, which were more suitable for water filtration and seed-derived germination; this was achieved by aerating the soil, along with tilling, followed by tuber thinning (Ens *et al.*, 2017).

Translocations of plants (entire plants, vegetative parts, or reproductive parts) by First Nations people as gifts or for trading helped to increase species patch densities, diversity, and proximity to some Indigenous occupation sites. Valuable groves of fruit and nut trees in the tropical rainforests of eastern Australia have been discovered near First Nations people's camps and ceremonial grounds. For instance, there is a relationship between red cabbage palm (*Livistona mariae*) in a central Australian riverbed and the mataranka palm (*Livistona rigida*) in the Top End. It is estimated that *Livistona mariae* had been translocated in the late Pleistocene, around 15,000 BP (Before Present, where 'Present' refers to AD 1950) (Kondo *et al.*, 2012).

Highly desirable species like root food species were extensively harvested and managed to preserve and increase the population. There were some protocols for harvesting fruits, including taking portions instead of whole crops and avoiding damage of unripe fruit, and breaking plant limbs (Ens *et al.*, 2017).

For wetlands management, First Nations people built weirs, diversions, ponds, traps, and constructed low-lying areas designed to flood seasonally. Even soaks, rock holes, and springs in arid regions were manipulated. Flow systems played an important role as a driver of river and floodplain wetlands with a higher diversity of species after temporary flooding and structural change, with sustained flooding.

First Nations people used fire as a land management tool to enhance the availability of plant and animal food resources, support hunting, and maintain walkable access to country (Bliege Bird *et al.*, 2024; Ens *et al.*, 2017; Kimber, 1983; Latz, 2007). For example, regular burning suppressed species that were sensitive to fire, often woody plants, while encouraging the growth of faster-growing, fire-tolerant vegetation.

Another purpose was to decrease landscape fuel loads that might initiate uncontrolled wildfire. For example, the Martu people of the Western Desert used strategic burning practices to manage vegetation and support the availability of food plants (Bliege Bird *et al.*, 2024; Fletcher *et al.*, 2021). Regular fire practices in the spinifex (*Triodia* spp.) savanna of the Western Desert have been shown to enhance plant diversity and reduce the risk of large wildfires. Within several decades of colonisation, the cessation of Indigenous burning practices resulted in substantial landscape transformations. The forest structure in the wet tropical rainforests of Queensland has faced major changes due to Indigenous fire management (e.g., its boundaries). Over thousands of generations, Indigenous people used skillful and persistent burning to shape a vegetation mosaic of rainforest, eucalypt savanna, and grassland in the mid-east coast subtropics (Ens *et al.*, 2017).

A recent example of fire-stick farming is the Kaurna Kardla Parranthi Cultural Burn, held in May 2021 in Adelaide's Park Lands. The Kaurna people are the Traditional Custodians of the Adelaide Plains, and this event marked the first public cultural burn in a capital city in over 240 years. It reflects the revival of Indigenous land management practices after a long disruption caused by colonisation (Figure 1.1). Similar cultural burning practices are being revived across Australia as part of broader efforts to reconnect with traditional knowledge systems.

Figure 1.1. Kaurna Cultural Burn in Adelaide Park Lands (2021). Kaurna community conducting a cultural burn in the Adelaide Park Lands, May 2021—the first public cultural burn in a capital city in over 240 years.

According to Drake et al. (2021), archaeological and ethnobotanical evidence shows that First Nations people's seed consumption, though not universal, was widespread—especially in arid and semi-arid regions of Australia. The earliest known seed grinding activity is dated to around 65,000 years ago, with more substantiated evidence from grindstones at Cuddie Springs in New South Wales dating back 30,000 years. These tools, bearing starch residues and distinctive wear, suggest a long-standing tradition of seed processing (Figure 1.2).

Preparing native grains, such as *Panicum* spp., involved several steps, including harvesting, threshing, winnowing, and grinding—each requiring both technical skill and cultural knowledge. Although threshing was one of the crucial steps, it is not well documented in historical records. In Central Australia, for instance, First Nations people used varied threshing methods based on the species and local conditions. For *Panicum* species, seed heads were often rubbed between the hands, trampled in a hole with the aid of pebbles, or beaten with sticks to loosen the seeds (Cane, 1987; Latz, 1995; Pattison *et al.*, 2023). These techniques helped the efficient separation of seeds with minimal damage, and some remaining husk parts were even retained to enhance the fibre content of the flour. Another example is when *Astrebla* on the Narran River was sometimes harvested while still green (i.e., not fully dried), fired, and pounded to release the seeds (Parker, 1905; Pattison *et al.*, 2023).

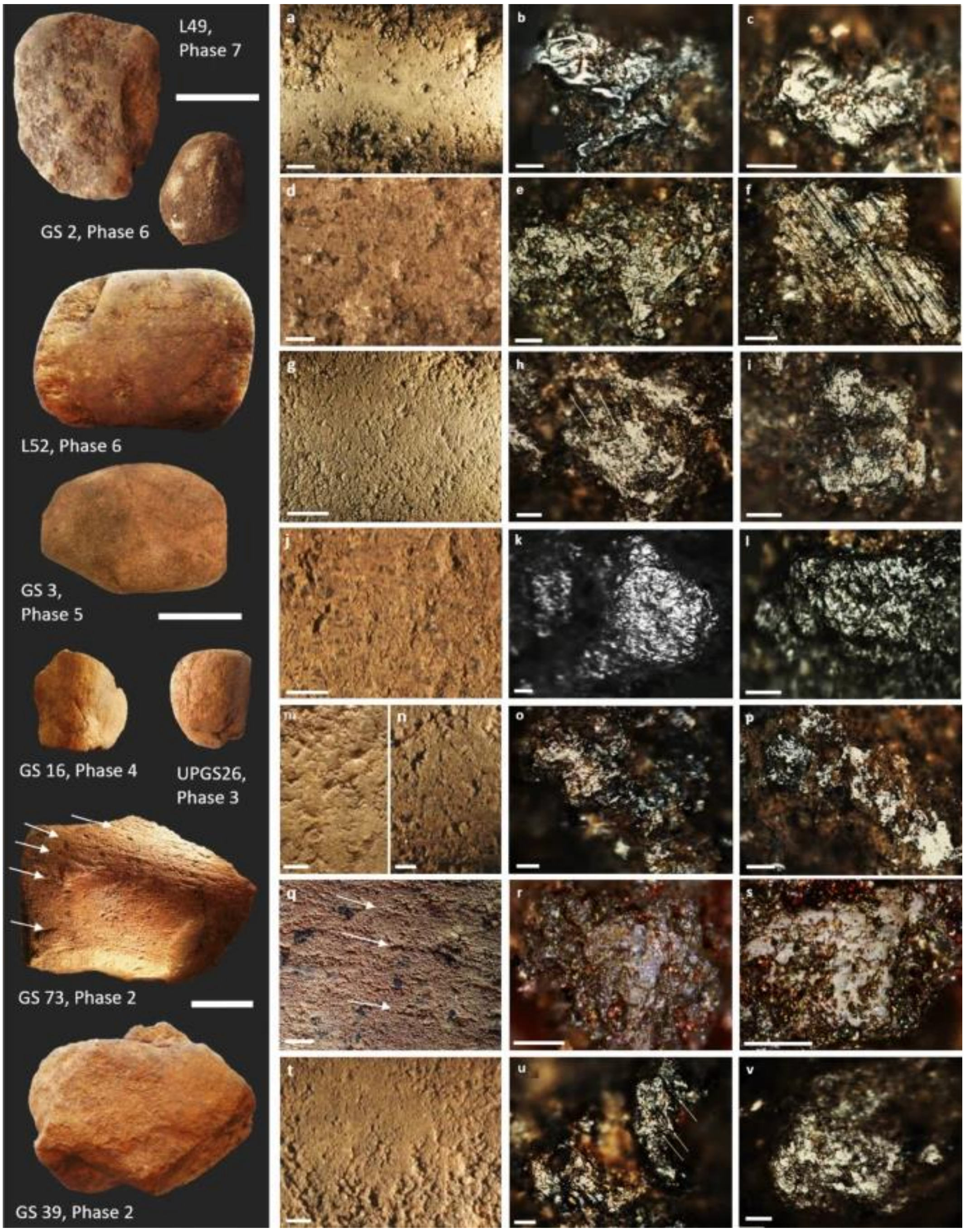

Figure 1.2. Grinding Stones and Micro-Residues from Madjedbebe. Examples of grinding stones from Madjedbebe, Northern Australia, with usewear and micro-residues consistent with plant and seed processing from Phases 7–2. Phases represent archaeological layers from different periods of site occupation; lower phase numbers indicate older layers. Labels (e.g., GS2 or L49) refer to specific excavation units within the site grid. The left-side images show grinding stones from excavation units such as L49, GS2, and GS16. Micrographs (a–v) display residues such as starch granules and plant tissues on the stones' surfaces. The findings offer clear evidence of long-term plant food processing by First Nations people. Scale bars: Artefact images = 5 cm; low magnification images vary: (a, g) = 5 mm; (m, n, q, t) = 2 mm; (d, j) = 1 mm; high magnification images = 0.05 mm (Hayes *et al.*, 2022)

1.1.2 Post-European Agricultural Development

Although a large area of Australia is arid or semi-arid, the agriculture sector has grown exceptionally well and produced well beyond the demands of the population. According to the Department of Foreign Affairs and Trade (DFAT, 2001), wool, wheat, beef, sugar, and butter dominated exports during the 1950s and 1960s. In 1969–70, wool, cereals, and meat comprised four-fifths of the value of agricultural exports. Over the same period, dairy products, tinned and frozen food, and animal feed increased from 16 to 39 percent of agricultural exports. For beverage exports, an increase from less than half a percent in 1969–70 to over nine percent in 2003–04 was observed (Productivity Commission, 2005; Voconiq, 2023).

When European settlers arrived in Australia in 1788, their main challenge was to provide enough food. They brought their agricultural technology and traditional practices from England. Wheat, barley, and oats were among the first crops brought to Australia with the First Fleet, helping to start grain farming. In the late 1800s, William Farrer began breeding wheat to make it grow better in Australia's tough conditions (Redden *et al.*, 2020).

Wheat production was a high priority for all the Australian colonies. The first farmers faced numerous challenges, given that they were not familiar with the Australian landscape and did not know where to farm. They also did not have any draught animals to help them. Another challenge was that farm labourers were scarce, and no farmyard manure was available. In New South Wales, early attempts to grow wheat were unsuccessful, particularly in the sandstone-derived soils of central Sydney and other coastal regions. These difficulties prompted a shift inland to more fertile areas, such as Parramatta and the Hawkesbury–Nepean River Valley (Henzell, 2007). Wheat continued to pose challenges, requiring imports from Tasmania and South Australia in some years. In addition, diseases like wheat rust threatened crop reliability. Following major outbreaks in the late 19th century, the New South Wales and Victorian governments appointed Australia's first plant pathologists—Nathan Cobb and Daniel McAlpine—in 1890 to investigate and manage these diseases (Ryley & Park, 2023).

The first successful grain harvest occurred in 1791–92 on the fertile flats of the Hawkesbury River, and after that, more than 70% of the colony's wheat and maize (by area) and all the oats and barley were cultivated in this area by 1810. The area of land under wheat and maize cultivation increased 10-fold by 1821 (compared to 1810). By 1840, almost half of the New South Wales wheat was produced in the valleys of the Hunter and Manning Rivers. By 1898, New South Wales was able to produce more wheat than its local needs and by 1911 displaced Victoria as the dominant wheat-producing state (Henzell, 2007).

Producing grains in Tasmania started around the convict settlement on the Derwent (1803) and Tamar (1804) Rivers. More fertile soil and regular rain, as well as less flooding, made agriculture more successful in Tasmania than in New South Wales. Indeed, the first Australian colony to be self-sufficient in wheat production was Tasmania (in 1815 or 1816). Although most of the wheat was produced in the south of the island in 1820, the balance had shifted to the north by 1850 (Henzell, 2007).

One of the challenges that the colonies faced was large hardwood trees, which occupied the locations that settlers wanted to farm. This was more of a problem in New South Wales than Tasmania because First Nations people’s burning practices had produced large areas of open country in Tasmania. Early Australian grain farmers were berated later for their poor farming practices, such as not using fertility-restoring fodder crops (legumes) in rotations. One of the reasons for this failure was the Australian environmental conditions, which negated the need for fodder crops. There was no need to house farming animals in winter (as there was in the U.K.), and there was an abundance of natural grazing (Henzell, 2007).

In South Australia, more fertile red-brown soil made agriculture more successful for the settlers who arrived in 1936. Farmers did not use any fertiliser for wheat production until the 1890s. Local wheat demand had been provided within seven years of farmers’ settlement. Mechanical harvesting played a key role in this success. After 1870, South Australia exported grains to the UK almost every year. Forty percent of Australian wheat was cultivated in South Australia in the 1880s. South Australia faced three major droughts between 1880 and 1903, which influenced the northern margins the most, leading to a low level of production. From the 1890s, farmers started using superphosphate fertilisers, along with bare fallowing, leading to an improvement in wheat production after those disastrous droughts (Henzell, 2007). The development of drought-tolerant grain varieties in the early 1900s helped expand grain farming in southeastern and southwestern Australia (Attard, 2008).

In the 1950s, farmers practiced legume-based pasture rotation with a cereal pattern. There was a growing attention to new crops such as grain legumes and oil seeds, as well as sheep farming, which led to diversification in South Australia. From 1990, farmers started applying nitrogen fertilisers, along with stopping intervening fallow or pasture, and rotating wheat with other grains (Henzell, 2007).

Victoria was settled one or two years before South Australia. The wheat industry's development was not rapid at first. During the 1880s and 1890s, planting fell in the Northern and Wimmera Districts but improved in the Mallee. Between 1890 and 1990, Victoria was dominant in wheat production among all states. Victoria played an important role in mechanical harvesting in Australia. For instance, harvesters that stripped and winnowed the grain were invented by James Morrow and Hugh Victor McKay in the 1880s and were manufactured in Ballarat and Melbourne. The Sunshine Harvester Works remained the biggest factory in Australia for many years (Henzell, 2007). During the 1850s, wheat farming in Victoria expanded significantly, particularly in the Wimmera region. This growth was influenced by population pressures from the gold rush, increased land availability, and improvements in farming methods and infrastructure (Ostapenko, 2013).

Developing effective technology for wheat production was slow in Queensland. During the Convict era, wheat production was not satisfactory in this state. After separation from New South Wales in 1859, almost 79 hectares were recorded under wheat cultivation in Queensland, mainly focused on Darling Downs around Warwick (Henzell, 2007).

When settlers arrived in 1829, they found farming in the southwest of Western Australia harder than anywhere else in Australia. Despite reliable rainfall, infertile soil (e.g., the sandy soil of the Swan coastal plain) was the main problem for wheat production. Farming started from a small area on the flood plains of the Swan River, which had fertile alluvial soil. Wheat production was not sufficient to meet local needs until the end of the century. Western Australia had to import its required grain and flour from South Australia. Population growth following the discovery of gold in the 1890s and the development of the state rail network were key factors in the establishment of the wheat industry in Western Australia. Using fertilisers, especially superphosphate, in Western Australia began in 1894 due to extremely infertile soil, leading to successful wheat production. In the 1930s, plans to expand a land development scheme toward the town of Southern Cross in Western Australia were abandoned due to natural soil salinity. This was later exacerbated by dryland salinity, which had damaged 0.9 million hectares of the state's land by 1992 (Henzell, 2007).

1.1.3 Contemporary Australian Crop Production

Cereals are both staple foods and key commodities in international trade. In Australia, wheat, barley, oats, sorghum, and rice (in some years) contribute notably to both domestic consumption and international trade (Figure 1.3). Wheat is the main grain produced in

Australia, with a production of approximately 26 million tonnes (Mt) in the 2023–2024 marketing year, equal to about 3.3% of the global total of 789 Mt. This was a decrease from the previous year's production of 41 Mt. Despite this fall, Australia remains a major wheat exporter and exports much of its wheat to Asian markets, especially Indonesia (NSW Department of Primary Industries, 2024; U.S. Department of Agriculture, 2023).

Barley was the fourth-largest cereal in global production after maize, rice, and wheat between 2010 and 2023. Australia's barley production is forecast to increase by 13% to 12.2 Mt in 2024–2025, which is 7% above the 10-year average, according to the Australian Bureau of Agricultural and Resource Economics and Sciences (ABARES, 2024).

Australia was the third-largest oat producer globally in 2023–2024, with a production of approximately 1.02 Mt, following Russia and Canada (Jain *et al.*, 2025; Statista, 2024).

According to the ABARES Report (2024), favourable weather conditions are expected to raise Australia's sorghum production by 9% to 2.4 Mt in 2024–2025. The United States is expected to produce 8.73 Mt of sorghum in 2024–2025, which is the largest amount globally and makes up 14% of world production (U.S. Department of Agriculture, 2024).

Rice production in Australia for the 2024–2025 season is forecast to decrease by 17% to 518,000 tonnes (0.518 Mt), according to the September 2024 Australian Crop Report by the Australian Bureau of Agricultural and Resource Economics and Sciences (ABARES, 2024). China and India are the world's top rice producers, each expected to produce approximately 145 Mt in the 2024–2025 marketing year and to contribute about 27% of global production (U.S. Department of Agriculture, 2024).

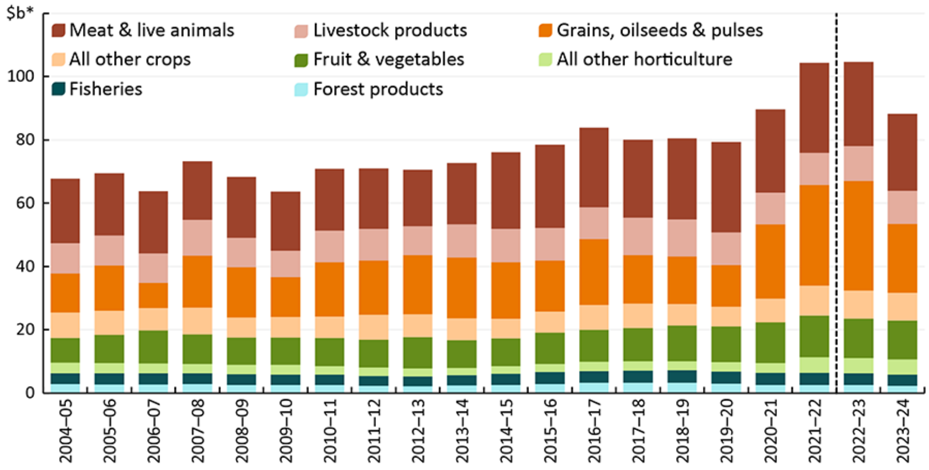

Figure 1.3. Value of Australian agricultural production by commodity group from 2004–05 to 2023–24. Values are in billions of Australian dollars ($b). The vertical dotted line indicates the division between historical data (2004–05 to 2022–23) and forecast data (2023–24) (ABARES, 2025).

1.2 The Role of Cereal Grains in Australian Food Systems

1.2.1 Nutritional and Dietary Importance

Cereal grains play an important role in the Australian diet, serving as a primary source of energy, dietary fibre, and essential nutrients such as B vitamins, iron, and magnesium (Grain & Legumes Nutrition Council, 2010; Reynolds *et al.*, 2019; Wu *et al.*, 2015). Wheat, particularly in the form of bread and other baked products, is a staple food in most Australian households. Oats and barley are less widely consumed than other cereals but provide important soluble fibre, including beta-glucan, which helps lower blood cholesterol levels. Public health recommendations in Australia encourage the consumption of wholegrain cereals as part of a balanced diet, citing their benefits in reducing the risk of chronic diseases such as cardiovascular disease, type-2 diabetes, and colorectal cancer (Liu *et al.*, 2024). As a result, cereal grains form the foundation of national dietary guidelines and are valued not only for their caloric contribution but also for their wider health benefits (Grains & Legumes Nutrition Council, 2023a; National Health and Medical Research Council, 2013; Wu *et al.*, 2015).

1.2.2 Food Industry and Processing

Cereal grains underpin the Australian food industry, forming the basis of flour, breakfast cereals, baked goods, and beverages. Wheat remains dominant due to its unique gluten proteins, which enable dough elasticity and improve bread-making quality, as well as pasta production. Malted barley is primarily used in malting for brewing and distilling, where its enzyme activity supports fermentation. Oats are widely processed into rolled and instant forms, with β-glucan recognised for cholesterol-lowering and digestive health benefits, making oats highly attractive for health-oriented products (Grains & Legumes Nutrition Council, 2023b; O'Hara *et al.*, 2013). Sorghum, once largely confined to livestock feed, has more recently entered Western human food markets, particularly gluten-free products, due to its antioxidant activity, fibre content, and potential digestive health benefits. These shifts illustrate how expanding nutritional and functional knowledge can transform the role of underutilised grains. However, Australian native grasses—despite their potential nutritional and functional attributes— remain underexplored in food processing contexts, leaving a critical gap this thesis seeks to address.

1.2.3 Market Preferences and Consumer Trends

Australian consumers have increasingly shown a preference for healthier and more diverse grain options, leading to a surge in demand for wholegrain breads, high-fibre breakfast cereals, and gluten-free alternatives such as sorghum and millet in recent years. This trend is strongly driven by awareness of the health benefits of whole-grain consumption, including improved digestion and a reduced risk of chronic diseases. (Birch *et al.*, 2023; Butcher, 2025; Jenifer *et al.*, 2023; Williams *et al.*, 2024b). Interest in ancient grains such as quinoa and amaranth also highlights consumer preference for products with strong nutritional profiles, environmental sustainability, and distinctive flavours. However, despite their long history of use by Indigenous Australians, native grains have not yet been incorporated into mainstream markets. The absence of detailed scientific evidence on their composition, processing functionality, and potential applications limits industry confidence in adopting them. Addressing this knowledge gap is a central aim of the present thesis, which investigates the morphological, biochemical, and functional properties of selected Australian native grains in the context of current consumer and market trends.

1.2.4 Food Security and Resilience

Cereal grains support food security in Australia due to their long shelf life, versatility, and high energy content. Growing climate variability and ongoing supply chain challenges highlight the importance of reliable grains such as wheat, barley, oats, and sorghum for both domestic use and export stability. Expanding cereal production to include native grains can enhance the resilience of the agricultural sector by reducing reliance on a narrow group of major crops and enabling better adaptation to environmental stresses. Increasing local grain diversity not only supports ecological sustainability but also strengthens food security by creating a more flexible and robust food system capable of withstanding global challenges such as climate change, market fluctuations, and supply chain disruptions (Drake *et al.*, 2021; Hughes *et al.*, 2015; Lopes *et al.*, 2023).

1.3 The History of Grain Use Among First Nations People

It is estimated that people migrated from Africa and Southeast Asia to Australia 47,000–60,000 years ago and later became the ancestors of today's First Nations people. Over thousands of years of experience, First Nations people managed native plants and animals to support food security (AgriFutures Australia, 2023; Clarkson *et al.*, 2017; Drake *et al.*, 2021; Pascoe, 2024; Redden *et al.*, 2020). Although the lifestyle of First Nations people has changed a lot over the last two centuries, traditional foods and cookery techniques continue to figure in Indigenous Australian lives (Dyson, 2006).

More than 1,100 species of perennial grasses endemic to Australia have been identified. The grains from these species have provided an essential part of the traditional Indigenous diet for millennia. Many of these plants are deep-rooted and tolerant of high temperatures and drought (Chivers *et al.*, 2015).

First Nations people developed different pre-treatments to prepare seeds before making pastes suitable for food production. They started by separating grass seeds by hand into wooden dishes. The next step was dehusking the seeds by rubbing them in pits. The seeds were then cleaned in winnowing trays and crushed into flour. To enhance the quality of grinding, pre-treatments were applied to the seeds, including parching, soaking, or cracking. It was common to sun-dry seeds before grinding them rather than soaking and grinding them wet (Mildwaters & Clarkson, 2020). Some seeds, such as mulga, were scorched in hot soil before being ground and made into pastes used for baking seed cakes (Smith, 2013). First Nations people's preferred method for cooking was using hot ashes rather than boiling the food, as they did not have access to cooking pots (Brand-Miller & Holt, 1998).

Small staple cereal grains that have been used in most regions of the world since the agricultural revolution, which were mainly seeds of the family *Poaceae*, were not substantially consumed in Australia by First Nations people before European settlement. Archaeological evidence suggests that around 15,000 years ago, certain cereal grains (e.g., *Panicum* spp.) played a significant role in Australia's grassland regions, particularly in areas with regular river flooding (Brand-Miller & Holt, 1998).

Wheat, rice, maize, barley, rye, and oats were brought to Australia by English settlers in 1788. Since then, almost all grain production in Australia has been from these exotic annual cereal crops (Hatton & Nulsen, 1999; Bell *et al.*, 2010; Redden *et al.*,2020).

In recent years, interest in cultivating and commercialising Australian native grains has resulted in research efforts across academic and industry sectors to address production challenges and emphasise their ecological, nutritional, and cultural importance (Figure 1.4).

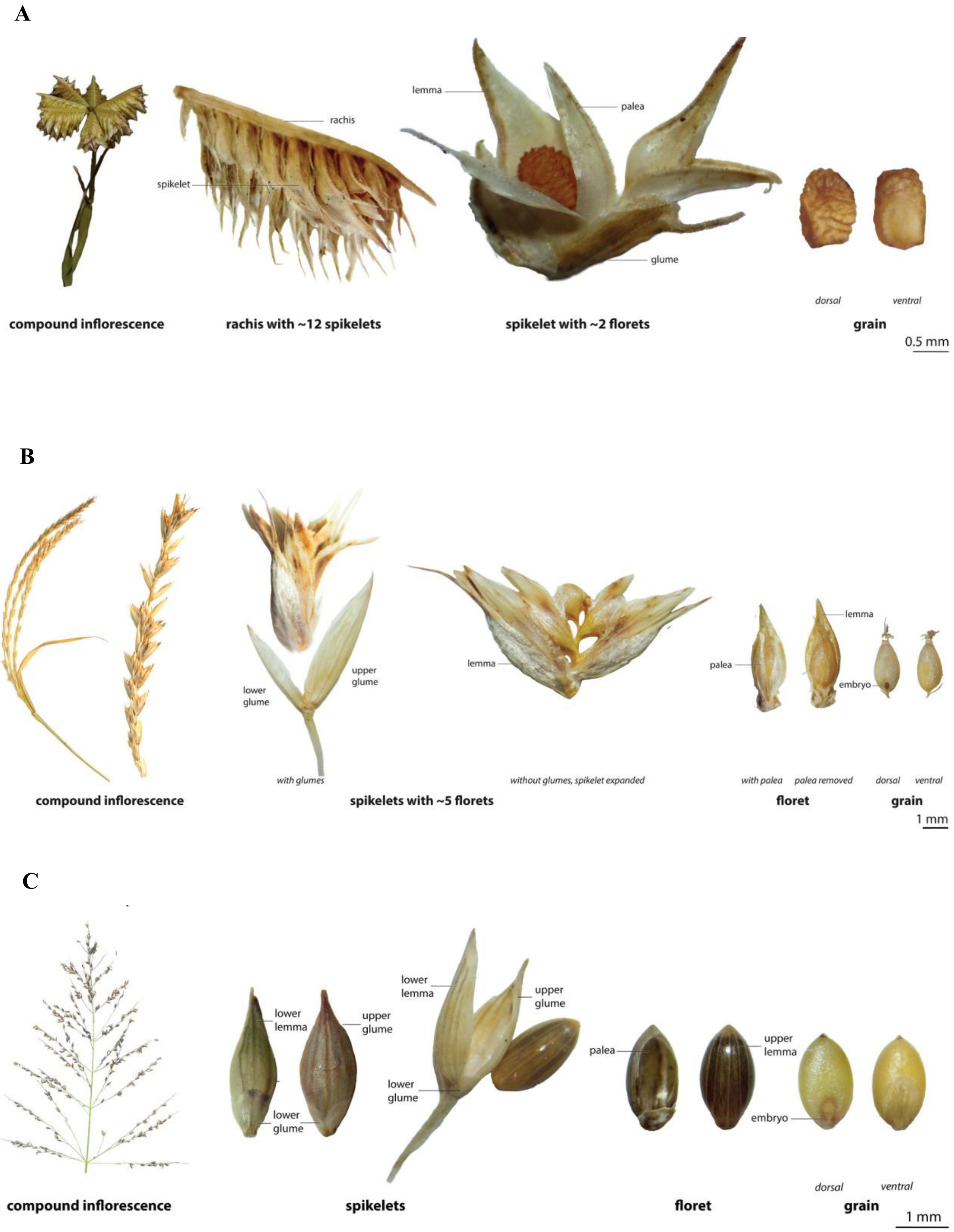

Figure 1.4 Morphological features of *Dactyloctenium radulans* (Button Grass) (A), *Astrebla lappacea* (Curly Mitchell Grass) (B), and *Panicum decompositum* (Native Millet) (C), showing inflorescences, spikelets, florets, and grains *(Pattison et al., 2023)* .

Four native grass species—Button Grass (*Dactyloctenium radulans*), Mitchell Grass (*Astrebla lappacea*), Native Millet (*Panicum decompositum*), and Weeping Grass (*Microlaena stipoides*)—were selected for this study due to their importance in traditional First Nations diets, their processing suitability, and their potential for use in modern food systems. These grains have distinct distributions across Australia (Figure 1.5).

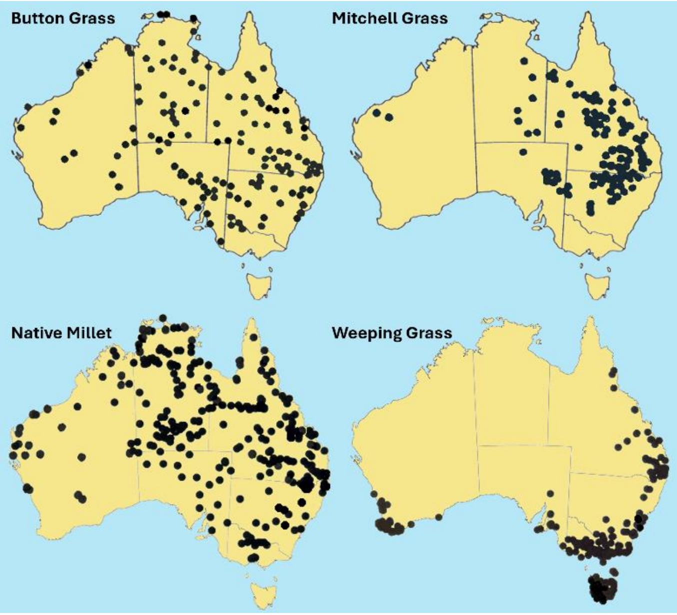

Figure 1.5. Distribution of four native Australian grasses based on verified records. The maps show recorded locations across Australia where each species has been observed (Australia's Virtual Herbarium, 2011; Australian Biological Resources Study, n.d.).

Dactyloctenium radulans (Button Grass) is a short plant, up to 20 cm in height. It has 3–10 spikes, 0.5–1.5 cm in length each, with densely crowded 2–4-flowered, 5-mm-long spikelets. This C4, annual (or short-lived perennial) grass is found in all states of Australia. Button Grass germinates in warm sandy soils, mainly in disturbed areas such as roadsides and stockyards (Cavanagh *et al.*, 2019; Jacobs *et al.*, 2008).

Astrebla lappacea (Curly Mitchell Grass) is a perennial C4 grass, up to 1 m in height, which grows in all mainland states of Australia except Victoria, with the main distribution in Northern NSW and southern Queensland. The loosely overlapping spikelets of Curly Mitchell Grass are 4–6–flowered, and 7–13 mm long (Cavanagh *et al.*, 2019; Jacobs *et al.*, 2008).

Panicum decompositum (Native Millet) is a perennial C4 grass, up to 1 m tall, with 2–flowered, 2.5–3.5 mm-long spikelets. Native Millet grows on good soils in all states of Australia (Jacobs *et al.*, 2008; Local Land Services, 2015).

Microlaena stipoides (Weeping Grass) is a C3 perennial grass, native to the eastern and southern regions of Australia. The height of the plant is 10–100 cm. The inflorescence is racemose, spike-like, and 5–25 cm long with 10–40 mm long, narrow spikelets. Weeping Grass is highly drought and acid tolerant (Cavanagh *et al.*, 2019; Jacobs *et al.*, 2008; Lamp *et al.*, 1990).

1.4 The Potential for Cultivating Native Grasses in Australia

1.4.1 Environmental and Agronomic Challenges in Staple Crop Production

1.4.1.1 Soil and Water Constraints

Global food demand continues to rise, with the world population projected to reach approximately 9.66 billion by 2050 under a medium-variant scenario (the United Nations' central, most likely projection) (United Nations, 2024). Meeting the nutritional demands of a growing global population will necessitate significant increases in food production, especially of staple crops.

Human conflicts, natural disasters, pests, and diseases contribute substantially to global losses in food production, storage, and distribution. Forest cover, protected areas, and land used for human settlements and economic infrastructure limit the land available for agricultural use. Moreover, constraints associated with a decrease in soil fertility impact agricultural productivity. Furthermore, the expansion of croplands results in soil degradation, pesticide and nutrient leaching, biodiversity loss, and carbon emissions, which are counted as poor agricultural practice (Varma *et al.*, 2024).

In Australia, soil acidification, dryland salinity, wind and water erosion, and poor soil carbon content are major agricultural challenges. Soil erosion leads to higher stream sediment loads, which impact infrastructure and riverine, estuarine, and marine ecosystems (Please, 2023; Xu *et al.*, 2002; Zhang *et al.*, 2022).

Dryland salinity, largely caused by replacement of deep-rooted perennials with shallow-rooted crops, restricts water and nutrient uptake and reduces yields (Hajkowicz & Young, 2005). Increased levels of salinity restrict the extraction of water and nutrients by plants, leading to increased levels of toxic ions such as sodium and chloride (Neave & Rayburg, 2006).

Soil acidification is a part of the soil development process and occurs slowly over time (a pH < 7 is considered acidic). However, increasing acidity can negatively affect crop production by reducing nutrient availability, limiting root growth, and increasing the solubility of toxic elements such as aluminium (Zheng, 2010). Old and weathered soils (like most of those in Australia) are more acidic than younger soils. Disruption of the carbon and nitrogen cycles, combined with fertiliser use and increased nutrient leaching, accelerates soil acidification (Hajkowicz & Young, 2005).

Various solutions have been suggested to address soil and water challenges, such as using smarter farming technologies, improving crop varieties, managing soil fertility more effectively, and making better use of underutilised land. Sustainable agriculture can also be advanced by improving pasture productivity, encouraging organic practices, and avoiding land-use changes that cause deforestation (McLennon *et al.*, 2021; Pesonen *et al.*, 2024; Sharma *et al.*, 2024). While these strategies are valuable, there is also growing interest in alternative crops such as native perennial grasses, which are naturally adapted to Australia's harsh soils and climate and may offer a more resilient solution (Bell *et al.*, 2022; Birch *et al.*, 2023; Pattison *et al.*, 2023; Pattison *et al.*, 2020).

The constraints above reduce the productivity of major cereals but highlight the potential role of Australian native perennial grasses, which are naturally adapted to such conditions. This thesis, therefore, investigates their morphological, biochemical, and functional properties of the grains from these grasses to better understand the suitability of these species as alternative grain crops.

1.4.1.2 Impacts of Climate Change

High temperatures and droughts are the primary effects of climate change and adversely influence food production. In southern hemisphere countries, agriculture is more vulnerable to climate change than in the northern hemisphere due to the greater frequency of extreme climate events (Govindaraj *et al.*, 2018). According to the Intergovernmental Panel on Climate Change (IPCC), if the rate of global warming does not change, we will face a 1.5 °C increase in global average temperature between 2030 and 2052 (Gorman *et al.*, 2020). This temperature rise is attributed to the release of greenhouse gases, mainly carbon dioxide, methane, and nitrous oxide (Korres *et al.*, 2016).

Water shortages and increasing demand for water in non-agricultural sectors are widely influential in Australian agricultural production. The Murray-Darling Basin (MDB), one of the main Australian food-producing areas, is an example of an area facing frequent drought, leading to a decrease in irrigated area. In 2017–2019, the MDB experienced a severe drought. Climate change predictions are that this area will be affected by an increasing drought frequency. Additionally, seasonal rainfall patterns will be altered by climate change (Qureshi *et al.*, 2013). Atmospheric circulation, including the El Niño Southern Oscillation, the Indian

Ocean dipole, and the southern annular mode, which cause large-scale regular droughts and floods, is an example of the influence of climate change in Eastern Australia (Zhang *et al.*, 2019). As a consequence of climate change, the decline in rainfall, along with increasingly frequency of extremely hot days, serves as a warning for increasing bushfire frequency and severity in Australia. An example is the Black Saturday fires in Victoria in 2009, when the maximum temperature in Melbourne hit 46.4° C, the highest ever recorded for the city, following a long drought (Yu *et al.*, 2020).

To cope with these adverse climate effects, some strategies are suggested. They include climate change adaptation in agriculture, which requires both technological solutions (new cultivars, more effective farming technologies, etc.) and non-technological solutions (markets, insurance, social networking, etc.). The development of major crop varieties resilient to heat and drought, the reintroduction of "unremembered" native plants, and improvement in support for agricultural research on native plants grown by smallholder farmers are also found to be effective. In semi-arid regions, planting trees and hedgerows, changing cropping patterns, trying new crops, and using crop rotation can help reduce erosion caused by heavy rain and wind (Govindaraj *et al.*, 2018).

1.4.2 Nutritional and Functional Potential of Indigenous Grains

In most major cereal grains, carbohydrates, primarily starch in the endosperm, make up approximately 56–74%, protein ranks as the second most abundant macronutrient (~8–11%), fibre is concentrated in the bran (~2–13%), and lipids comprise ~2–4% dry weight of the grains. The nutritional composition of cereal grains makes them well-suited for diverse food applications. High-carbohydrate grains, for instance, are well-suited for calorie-rich products, while grains higher in fibre, B vitamins, and antioxidants appeal to health-conscious consumers (Garutti *et al.*, 2022; Koehler & Wieser, 2013; Poutanen *et al.*, 2022).

Although cereals such as sorghum, rye, oats, and teff are produced in significant quantities, they are often categorised as 'minor cereals' due to their lower global production and market presence compared to staples like wheat, rice, and maize. Despite this, they offer unique nutritional potential and contribute to dietary diversity. Fibre, minerals, and bioactive compounds are often higher in minor cereal grains. As modern markets increasingly prioritise healthy foods over core nutrients alone, this shift is reshaping the grain industry, with growing interest in minor cereals due to their potential in healthy cereal-based products (Wang *et al.*, 2023).

Despite limited research on Australian native grains, available studies have reported promising nutritional traits. For example, Curly Mitchell Grass was found to contain appreciable levels of dietary fibre and protein (Foster *et al.*, 2010). More recent analyses have highlighted high protein levels, bioactive phenolic compounds with antioxidant properties, and essential minerals such as calcium, iron, zinc, magnesium, and potassium across several species (Birch *et al.*, 2023; Brand-Miller & Holt, 1998; Cowley *et al.*, 2023; Foster *et al.*, 2010; Miller *et al.*, 1993; Rahman *et al.*, 2021; Smith & Smith, 2003; Wurm *et al.*, 2012). However, these studies remain limited in scope, often focusing on individual species or reporting only basic compositional traits, with little information available on functional and processing properties. This thesis builds on these preliminary findings by examining the morphological, biochemical, and functional characteristics of four native grain species in comparison with domesticated cereals, thereby addressing these gaps and providing a more comprehensive understanding of their potential as food crops.

1.4.3 Indigenous Knowledge Systems and Cultural Contexts

By observing the land, plants, animals, and climate, First Nations people in Australia developed sophisticated knowledge systems over tens of thousands of years. These systems are transmitted across generations through storytelling, songlines, and cultural practices. The systems value responsibility and care for Country above ownership, as land is regarded as an integral part of the community and culture (Curran *et al.*, 2019; Drake *et al.*, 2021).

Cultural burning, or fire-stick farming, is a form of ecological knowledge that uses low-intensity, planned fires to manage the land by promoting plant growth, reducing bushfire risk, and supporting hunting and seed harvesting. For thousands of years, Indigenous communities have used fire in this way and revealed a detailed understanding of seasonal patterns and ecosystems (Horton, 1982; Wilman, 2015).

Traditional knowledge includes the use of native grains, roots, and seeds for food; species such as Native Millet (*Panicum decompositum*) and Kangaroo Grass (*Themeda triandra*) served not only as important sources of nutrition but also had roles in trade, ceremony, and daily life. The processing of these grains—harvesting, winnowing, grinding, and baking—shows a sophisticated understanding of food preparation, even in harsh environments (Drake *et al.*, 2021).

This knowledge is being revalued and reconsidered in response to the growing interest in sustainable and culturally respectful food systems. Incorporating Indigenous voices and leadership in native grain research is essential to honour ethical standards and ensure the respect and protection of cultural knowledge (Lopes *et al.*, 2023).

1.4.4 Challenges and Limitations of Native Grass Cultivation

1.4.4.1 Agronomic Constraints and Yield Variability

For thousands of years, repeated selection and breeding have produced high-yielding annual cereal crops adapted to specific environments. In the 20th century, agricultural development—driven by greater access to agrochemicals and irrigation—further improved these cropping systems. During this time, increasing grain yield became a central objective and a key factor in the successful commercialisation of crops (Chapman *et al.*, 2022).

Modern farming systems continue to prioritise yield maximisation, often through the cultivation of annual monocultures supported by external inputs. Although this model has improved productivity and supported global food security, it has also led to long-term soil degradation, a decline in soil organic matter, and disruption of hydrological cycles. The replacement of deep-rooted perennial vegetation with shallow-rooted annuals has reduced groundwater recharge, increased surface runoff, and elevated the risk of drought and erosion (Newell, 2021).

Although Australian native grasses have a strong potential for grain production due to their ecological resilience, their adoption is limited by yield variability and lower productivity compared to commercial cereals. While native species yield between 8 and 2,200 kg/ha, depending on species and conditions, commercial wheat can produce up to 10 tonnes per hectare in high-rainfall areas (Cole & Johnston, 2006; Drake *et al.*, 2021), while the variable performance and lower yields of native grains pose significant challenges for their integration into conventional food systems.

1.4.4.2 Harvesting and Processing Difficulties

The unique morphology of Australian native grains, along with the absence of suitable machinery, limits their harvesting and processing efficiency.

Native grasses such as Native Millet (*Panicum decompositum*), which are characterised by small seed size, variable maturation times, and protective husks (lemma and palea), present challenges for harvesting and reduce the effectiveness of industrial equipment designed for uniform, large-seeded cereals like wheat and barley. Consequently, reliance on manual labour or specialised machinery leads to reduced overall efficiency (Lopes *et al.*, 2023).

Post-harvest processing of native grains is comparably challenging. Due to the wide variation in seed sizes and shapes, removing plant debris and chaff often involves a complex and extensive cleaning process. Modern techniques must be adapted or developed to ensure efficient processing and compliance with food safety and quality standards, due to the limitations of traditional methods in commercial scaling (Pattison *et al.*, 2025).

Despite the challenges mentioned above, research continues to focus on developing specialised equipment and processing methods suited to native grains. Collaborations between Indigenous communities, researchers, and industry stakeholders are crucial in creating sustainable and culturally appropriate solutions that respect traditional knowledge while advancing commercial viability (Pattison *et al.*, 2025).

1.4.4.3 Market Integration and Economic Viability

Despite their nutritional and environmental advantages, native grains face multiple challenges to commercialisation. These include challenges related to sensory acceptance, processing limitations, infrastructure, and limited consumer awareness. For example, grains such as Native Millet (*Panicum decompositum*) often have darker colours, coarser textures, and stronger flavours than domesticated cereals, which can negatively affect consumer acceptance in mainstream markets (Jenifer *et al.*, 2023).

A range of strategies can be implemented to improve the commercial viability and market integration of native grains:

- Partially incorporating native grain flours with widely accepted domesticated grains (e.g., wheat) can help maintain familiar sensory attributes while diversifying product offerings.
- Processing efficiency and cost-effectiveness can be improved by optimising equipment to suit the specific morphological and functional characteristics of native grains.
- Methods such as fermentation, debittering, and milling modifications can enhance flavour, mouthfeel, and overall consumer acceptance.
- Collaborative efforts involving Indigenous enterprises, grain producers, and food industry stakeholders can enable scalable production and strengthen supply chain reliability.
- Marketing efforts that highlight the cultural and environmental value of native grains can help present them as high-quality, distinctive products.

- Clear and transparent labelling that communicates nutritional qualities, gluten-free status, and ecological benefits can build consumer trust and stimulate demand.

The full potential of Australian native grasses in food systems can only be realised by addressing the research needs and opportunities highlighted by these combined factors.

1.5 Grain Physicochemical Factors in Commercialisation

1.5.1 Morphology and Structure

1.5.1.1 Grain Size

According to the International Rice Research Institute (IRRI) breeding programs, rice grain size is classified based on length as follows: short (≤5.50 mm), medium/intermediate (5.51–6.60 mm), long (6.61–7.50 mm), and very long (>7.50 mm) (Cuevas *et al.*, 2016) .

Grain size plays an important role in yield by influencing seedling vigour. Although smaller seeds tend to emerge faster, they produce less shoot dry weight, resulting in a lower overall yield (Lafond & Baker, 1986; Whan *et al.*, 2014). Higher milling yields can result from seed size and other kernel traits, including bran thickness, the amount of germ tissue, and crease depth (Whan *et al.*, 2014) .

According to Jenifer et al. (2023), the small size of the grains of Native Millet (*Panicum decompositum*) resulted in a milling yield that was 4–10% lower compared to wheat under similar conditions. Among native grains, Weeping Grass, with its larger, elongated grains, may be well-suited to market preferences for higher milling yields and improved processing efficiency.

1.5.1.2 Grain Shape

Seed dimensions, namely seed length (SL), seed width (SW) and the ratio of length to width (L:W), largely determine seed shape. The size and shape of grains can influence heat and moisture transfer during the drying process. Due to the uniformity of heat distribution, drying more spherical grains is associated with a lower risk of incomplete drying and potential spoilage compared to grains of other shapes.

More spherical grains may result in higher flour yield and quality, with less mechanical damage compared to irregularly shaped grains. The shape of the grains also enhances storage efficiency during transport and handling. In more spherical grains, for example, the grains flow more easily, which reduces the risk of equipment becoming clogged (Sablani & Ramaswamy, 2003; Shrestha, 2020).

In rice, grain shape influences appearance, milling performance, cooking quality, and yield. In regions such as China, it also plays an important role in determining consumer preference and market value (Qiu *et al.*, 2012).

In native grains, more spherical shapes, such as those in Button Grass and Curly Mitchell Grass, can improve drying efficiency, storage handling, and reduce quality loss by facilitating flow and minimising equipment clogging. Elongated grains, like those of Weeping Grass and Kangaroo Grass, can meet specific consumer preferences, like the appeal of rice (Abedi *et al.*, 2023; Cowley *et al.*, 2023).

1.5.1.3 Grain Colour

Grain colour plays an important role in not only the visual appeal of the processed grain but also key quality attributes, including nutritional composition, antioxidant content, and processing characteristics (Liu *et al.*, 2010; Saini *et al.*, 2023). Colour variations can also impact consumer perceptions of freshness, flavour, and nutritional content. The dark colour associated with some dietary fibre sources can negatively impact the sensory appeal of food products (Heiniö *et al.*, 2016). The bitter or astringent taste in dark rye, greyish barley, brown rice, and sorghum (red, brown, or black) is attributed to the presence of polyphenols (Baik & Ullrich, 2008).

The relatively dark colour of Native Millet products (e.g., flatbreads) influences consumer preferences, as many customers find the colour unappealing. Improving market acceptance requires educating consumers about the benefits of native grains and applying strategies like blending native grain flour with other flours (Jenifer *et al.*, 2023).

1.5.1.4 Bran Composition

Cereal bran is a multi-layered structure made up of the pericarp, testa, and aleurone layer—outer grain components that provide protection and are rich in dietary fibre, phenolic compounds, and minerals. In some cases, it consists of adherent residual endosperm tissues. Cell walls in these layers contain various components, mainly polysaccharides (e.g., cellulose and heteroxylans), proteins, phenolic acids, and lower levels of lignin. In wholemeal cereal flour, bran components—particularly arabinoxylans, a non-cellulosic dietary fibre—affect various rheological properties. In most cereals, the thin cell walls surrounding the starchy endosperm and aleurone layer are primarily composed of arabinoxylans, which make up approximately 60–70% of the cell wall structure. Arabinoxylans are the major endosperm cell-wall polysaccharides in wheat/rye, but are much less abundant in the β-glucan–rich oats and barley, and relatively higher again in rice (cell-wall basis) (Fincher & Stone, 1986).

Cell wall components such as arabinoxylans influence the hydration and rheological properties of cereal doughs. Water-extractable arabinoxylans can increase water absorption and viscosity, whereas water-unextractable forms may interfere with gluten development and reduce dough stability (Courtin & Delcour, 2002). Depending on their solubility and structural characteristics, arabinoxylans may therefore improve or impair dough extensibility and bread quality (Izydorczyk & Biliaderis, 2007).

Native grains are often used in wholemeal form, which can result in flour with a higher bran content, due to their smaller size compared to domesticated grains (Jenifer *et al.*, 2023). Understanding the nutritional and functional properties of native grain bran is essential to unlocking their commercial potential for Indigenous enterprises, despite the limited research available. These attributes play an important role in enhancing processing efficiency and contributing to consumer appeal.

1.5.1.5 Endosperm Structure

Endosperm composition plays an important role in determining milling performance and flour yield in cereal grains. In hard wheat varieties, the hard endosperm structure allows the bran to separate more efficiently from the endosperm, which results in higher flour extraction rates compared to softer wheat (Edwards *et al.*, 2008). Functional properties closely relate to grain composition, including protein, starch, lipid, and fibre content.

In wheat, milling efficiency is affected by the size distribution of starch granules in the endosperm, with A-type granules (the larger) measuring between 10 and 35 µm in diameter. Although they account for less than 10% of the total number of granules, they contribute more than 70% of the total starch mass. B-type granules (5.3–10.0 µm) and C-type granules (less than 5.3 µm) are smaller and, although they make up most starch granules by number (over 90%), they contribute less than 30% of the total starch mass. The smaller size of B- and C-type granules results in a higher surface area-to-volume ratio, which may facilitate stronger interactions between starch and other grain components such as proteins and lipids. These interactions can reinforce the structural integrity of the granules, potentially increasing hardness. However, increased hardness does not necessarily improve milling yield. While harder grains may produce finer flour under controlled milling, they often require more energy to fracture and may lead to higher starch damage, which can reduce yield and affect flour quality (Edwards *et al.*, 2008).

The protein matrix within the endosperm plays an important role in determining flour properties. The interaction between proteins and starch granules, particularly in harder cereals, is believed to influence starch digestibility and flour behaviour during milling. In hard endosperm flour, the protein network binds tightly to starch and increases starch damage during milling, which enhances starch digestibility in raw flour. However, during cooking, this protein-starch interaction may reduce water absorption and starch gelatinisation and lead to slower starch digestion. This shows that the protein composition of the endosperm affects not only the digestibility of flour but also its performance during processing.

Spherical lipid bodies measuring 0.2–2.0 µm in diameter are found within the endosperm, with the highest concentration in the sub-aleurone region. These lipid bodies resemble spherosomes and contain triglycerides, which are surrounded by a monolayer of proteins and diacyl phospholipids. According to research, elevated lipid levels are commonly linked to smaller starch granules, particularly B- and C-type. The presence of lipids on smaller granules is thought to influence the mechanical properties of the grain by forming complexes with amylose or embedding within the starch matrix. These interactions can restrict swelling and water absorption, thereby increasing granule rigidity and overall grain hardness, which may in turn affect milling performance (Edwards *et al.*, 2008).

The endosperm composition of Australian native grains, such as Weeping Grass (*Microlaena stipoides*) and Curly Mitchell Grass (*Astrebla lappacea*), displays unique characteristics that may help their commercial applications. In both grains, starch granules are slightly smaller than those in domesticated rice (*Oryza sativa*), where granules typically range from 3 to 5 µm (Shapter *et al.*, 2008). Granule sizes in Weeping Grass (1–4 µm) and Curly Mitchell Grass (2–5 µm) are comparable to wheat C-type granules, which enhance milling efficiency and mechanical properties due to their high surface-area-to-volume ratio. Although direct evidence is limited, the smaller granule size of these native grains may support easier processing and enhance their suitability for milling and product development (Shapter *et al.*, 2008).

1.5.2 Ease of Threshing and Processing

Threshing is the process of separating edible grain from husks and straw, and effective methods result in a cleaner, more nutritious product that meets consumer demand for high-quality, nutrient-rich grains (Wrigley *et al.*, 2015).

First Nations people adapted and refined traditional threshing techniques to accommodate the distinct characteristics of each grain species. In Native Millet (*Panicum decompositum*), the palea and lemma are tightly bound to caryopsis, which led to their traditional inclusion in the final flour and contributed to its higher fibre content. These traditional practices highlight the need for mechanised threshing processes adapted to the unique characteristics of different native grain species (Abedi *et al.*, 2023; Latz, 1995).

1.5.3 Shelf Life and Storage Stability

Grain shelf life and, by extension, commercial viability, are strongly influenced by lipid content and enzyme activity in cereal grains. Domesticated grains like oats and wheat have relatively high concentrations of polyunsaturated fatty acids (PUFA) and active lipases, which can release free fatty acids (FFA) under poor storage conditions. The oxidation of these FFAs leads to rancidity, off-flavours, and the formation of bitter compounds. Such degradation negatively affects grain quality, limits its shelf life, and decreases its marketability (Alpuche-Solís & Paredes-López, 2000).

Based on recent studies, selected native grain species contain higher total lipid levels, which can potentially decrease their shelf life. However, according to research on unspecified native grain species, levels of monounsaturated fatty acids (MUFA)—which are more resistant to oxidation than PUFA—are higher in these grains compared to wheat. The stability of MUFAs enables these native grains to maintain their quality for longer times and makes them well-suited for long-term storage and transport (Williams *et al.*, 2024a).

1.5.4 Allergenicity and Gluten-Free Potential

1.5.4.1 Allergenicity

At present, there is a lack of comprehensive research on the allergenic potential of these native grains. Anecdotal evidence suggests that Weeping Grass (*Microlaena stipoides*) is a low-allergen plant and may be appropriate for individuals with certain grass allergies. Nonetheless, comprehensive clinical studies are necessary to confirm the hypoallergenic properties of these grains and to ensure their safety for consumers with specific food allergies (Respiratory Care WA, 2024).

1.5.4.2 Gluten-Free Potential

Australian native grains are naturally gluten-free and act as promising alternatives for individuals with coeliac disease or non-coeliac gluten sensitivity (Butcher, 2025; Jenifer *et al.*, 2023). Unlike domesticated cereal grains such as wheat, barley, and rye, which contain gluten-forming proteins, native grasses belong to species that do not produce these protein fractions. Their gluten-free status, combined with favourable nutritional profiles rich in dietary fibre, protein, and bioactive compounds, makes these grains especially appealing for health-focused and specialty food products (Williams *et al.*, 2024b). Native grains can be incorporated into gluten-free flours, cereals, or baked goods—either alone or in blends—to enhance nutritional value while maintaining gluten-free standards.

In many countries, including Australia, regulatory frameworks require validated testing and certification before a product can be labelled as gluten-free, even if the grains have the potential to meet gluten-free standards (Coeliac Australia, n.d.). Further research and analytical validation—such as enzyme-linked immunosorbent assay (ELISA) or liquid chromatography–mass spectrometry (LC-MS)-based gluten assays—are necessary to confirm the complete absence of immunogenic gluten peptides in these species (Osorio *et al.*, 2019).

1.5.5 Functional Properties of Cereal Grains

Functional properties are physical and chemical characteristics that affect cereal grain flour's behaviour during processing and preparation in various food applications like baked products, thickeners, emulsions, and dough or batter. This highlights the importance of functional properties in selecting suitable grains for specific food products and in improving processing efficiency and consumer acceptance (Awuchi *et al.*, 2019).

Key parameters used to assess functionality include water and oil absorption capacity, water absorption index, swelling capacity, foaming capacity, and bulk density. These measures offer valuable insight into the potential applications of cereal flours, especially when assessing underutilised species like Australian native grains.

1.5.5.1 Water Absorption Capacity (WAC)

Water absorption capacity refers to the ability of flour to retain water under limited centrifugation. It reflects interactions between flour components—such as proteins and polysaccharides—and water. High WAC is often linked to abundant hydrophilic constituents, particularly dietary fibre and denatured proteins (Kinsella & Melachouris, 1976; Sosulski, 1962).

Applications such as dough formation and baked products rely on WAC for effective water retention. In the absence of gluten, particularly in gluten-free formulations, a high WAC plays a key role in preserving moisture and supporting product structure (Furlán *et al.*, 2015; Sahin *et al.*, 2020).

Among Australian native seeds, wattle seeds are the most thoroughly studied for their functional properties. Although wattle seed is not a cereal grain, it is often included in discussions about native grains because of its similar food uses and nutrient composition. Wattle seed flour (from domesticated Australian *Acacia* species) has been reported to exhibit a high WAC (Adiamo *et al.*, 2024). In contrast, there is limited published information on the WAC of most Australian native grain flours, which creates a gap in the current understanding of their functional characteristics. Native Millet (*Panicum decompositum*) is one example. Although formal WAC data for Native Millet are lacking, a study on flatbread preparation found that a 50:50 blend with wheat flour required 168 mL of water per 100 g of flour, close to the 167 mL used for 100% wheat flour (Jenifer *et al.*, 2023).

1.5.5.2 Oil Absorption Capacity (OAC)

Protein structure plays a major role in oil absorption—the ability of flour to bind oil—with non-polar side chains, lipophilic amino acids, and porous flour structures all contributing to higher OAC. OAC is essential for products like sausages, baked snacks, or powdered soups, where oil retention affects texture, mouthfeel, and flavour delivery (Wang, 2020) .

Studies on the OAC of Australian native grains remain limited. One of the few available investigations is on wattle seed flour, which has shown relatively low OAC despite exhibiting high WAC (Adiamo *et al.*, 2024).

1.5.5.3 Swelling Capacity

Swelling capacity is an increase in flour volume after hydration. The capacity of starch and fibre to absorb water and expand depends on factors such as flour fineness, fibre type, and the ratio of soluble to insoluble components (Burešová *et al.*, 2023; Wang *et al.*, 2024).

Swelling capacity helps improve the structure of gluten-free products and enhances the texture of baked goods by increasing volume, porosity, and chewiness (Cappelli *et al.*, 2020; Konik *et al.*, 1993).

There is little reported research on the swelling capacity of Australian native grains. This gap limits our understanding of their functional properties and their potential use in food applications.

1.5.5.4 Foaming Capacity

Foaming capacity is the ability to form and stabilise gas bubbles. In baking and confectionery, ingredients with high foaming capacity support better aeration, increased volume, and a lighter texture. For example, cereal flours or protein ingredients that form stable foams can improve crumb softness, shelf life, and overall appearance in baked goods (Siddiqua *et al.*, 2019).

In beverage production, foam is often critical to product quality. Beer foam stability depends on foam-stabilising proteins derived from malted grains, and brewers optimise processes to maximise these components. For instance, research confirms that wheat malts (used in wheat beers) tend to produce more abundant and stable foam than barley malts alone, owing to higher protein levels and foam-positive polypeptides (Combe *et al.*, 2013).

Foaming is strongly influenced by pH, which affects the charge, solubility, and structural properties of cereal proteins. At the isoelectric point—where proteins have no net charge and limited solubility—foaming capacity is typically at its lowest. Proteins remain more soluble and exhibit improved foaming performance under slightly acidic to alkaline conditions. However, extreme pH levels can cause protein denaturation or precipitation, leading to reduced formation. Based on the literature, a pH range of 6–8 is optimal for enhancing the foaming capacity of many cereal proteins (Olanca & Özay, 2010).

The functional properties of cereal protein fractions have a strong influence on foaming. Albumins and globulins are particularly effective, as they reduce surface tension and stabilise air bubbles, and play a key role in foam formation (Shen *et al.*, 2025). Prolamins and glutelins have distinct structures that influence their functional properties. Prolamins, such as hordein in barley, are rich in hydrophobic amino acids and tend to form flexible films at air–water interfaces, contributing to good foaming capacity. In contrast, glutelins have higher molecular weights and more disulfide bonds, which reduce foaming ability but enhance emulsifying properties due to stronger protein–protein and protein–lipid interactions (Wang *et al.*, 2010; Zhao *et al.*, 2011; Zhu *et al.*, 2021).

Polar lipids and surfactants in cereals (e.g., lecithins and glycolipids) can improve foaming by stabilising foam layers, while non-polar lipids tend to weaken foams and cause them to collapse. Phenolic compounds (such as polyphenols and tannins in sorghum and barley) can interact with proteins. At low levels, they may strengthen foam by cross-linking proteins, while higher concentrations or specific types can induce protein aggregation and reduce foaming, as observed in some specialty barley malts containing foam-negative polyphenols (Combe *et al.*, 2013).

1.5.5.5 Bulk Density

Bulk density is the flour mass per unit volume measured under loose and packed conditions. It reflects the particle size, shape, and compaction behaviour of the flour (Chandra *et al.*, 2015). Lower bulk density is associated with more air space and porosity, while higher density reflects finer or denser particles (Bala *et al.*, 2020). This property is relevant for packaging, transportation, and storage. It also affects mixing behaviour and expansion in extruded products (Ganesan *et al.*, 2021).

Native grains with smaller seeds and harder endosperm may produce coarser or more irregularly shaped flours, which could lead to lower bulk density compared to flours from more uniform and softer-textured cereals. According to Jenifer et al. (2023), Native Millet (*Panicum decompositum*) is characterised by small grain size, dense kernels, and a high proportion of bran. Although bulk density was not directly measured, these features suggest that Native Millet flour may have a lower bulk density than wheat flour. This interpretation relies on the grain’s physical and compositional characteristics, particularly kernel hardness and outer layer content.

1.5.5.6 Functional Properties and Commercial Potential

A clear understanding of the functional properties of Australian native grains is essential, as these characteristics directly affect processing behaviour, product texture, and consumer acceptance in modern food systems.

For example, high water absorption capacity (WAC) and strong swelling ability are useful in gluten-free doughs and other hydrated products, as they help retain moisture and improve texture. A moderate oil absorption capacity (OAC) can enhance the mouthfeel of plant-based foods by adding richness and supporting flavour delivery. Foaming capacity also plays an important role in baked and aerated products, where it contributes to volume and lightness, although its effectiveness depends on the type and structure of the proteins present. While low bulk density can make packaging and transport more difficult, it is advantageous in extruded foods because it supports better expansion and texture. In this study, Native Millet and Weeping Grass showed lower bulk density compared to Curly Mitchell Grass and Button Grass. This suggests that Native Millet and Weeping Grass may offer better expansion and textural qualities in extruded products, whereas the higher bulk density of Curly Mitchell Grass and Button Grass may limit their expansion potential.

The variability of these traits among native grain species suggests that each may be suited to different product applications. Functional testing provides a scientific foundation for product development and supports the case for diversifying the Australian grain market with more sustainable, native options.

1.6 Conclusion and Aims of the Project

Both Indigenous land management and European agriculture have shaped Australia's food systems through the use of cereal grains—both domesticated species like wheat and barley, and native grass seeds such as Native Millet and Weeping Grass, which are botanically classified within the cereal family. Although domesticated cereals such as wheat, barley, oats, rice, and sorghum continue to dominate commercial production, native grains are gaining recognition for their potential to support environmental resilience, cultural renewal, and nutritional diversity.

For over 60,000 years, First Nations people have actively managed Australia's landscapes by practising fire management, relocating species, and engineering wetlands. Native grasses played a crucial role in these systems. Both archaeobotanical findings and ethnographic accounts suggest that seed harvesting, threshing, and grinding were commonly practised throughout the continent, with methods tailored to specific ecological and cultural settings. However, colonisation disrupted these systems, and for more than two centuries, Australian agriculture has largely centred on a limited range of introduced annual cereal crops.

Today, global and national agricultural systems face urgent challenges. Climate change, soil degradation, water scarcity, and market volatility all threaten the sustainability of current food production models. Simultaneously, consumer preferences are shifting toward healthier, more sustainable, and culturally meaningful foods. Interest in the commercial potential of Australian native grains has grown in response to these changing circumstances. Many of these grains are perennial and deep-rooted, require minimal inputs, and provide promising nutritional benefits.

Despite their promise, native grains face significant challenges to integration within commercial systems. Agronomic limitations—such as yield variability, small seed size, and difficult threshing—limit scalability. Post-harvest processing remains underdeveloped, often requiring labour-intensive cleaning or new mechanical innovations. Additionally, market uptake is constrained by unfamiliarity, inconsistent sensory attributes, and a lack of infrastructure.

Several native grasses—including Native Millet (*Panicum decompositum*), Weeping Grass (*Microlaena stipoides*), Curly Mitchell Grass (*Astrebla lappacea*), and Button Grass (*Dactyloctenium radulans*)—have been identified as having traits that warrant further investigation. Preliminary reports suggest they contain favourable levels of protein, fibre, and bioactive compounds.

Variation in the physical and chemical properties of native grains suggests that a single, uniform processing approach will not be effective. Instead, grain-specific strategies should be developed based on processing needs, nutritional goals, and market applications. Understanding traits like endosperm structure, starch granule size, and protein composition will support targeted breeding or mechanical optimisation, while preserving the ecological and cultural integrity of these species.

Crucially, the future of native grains must be guided by Indigenous leadership and knowledge. As the original stewards of these landscapes and crops, First Nations people hold ecological, cultural, and culinary expertise that can inform both research and enterprise development. Ethical engagement and co-design processes will be key to ensuring that native grain commercialisation supports community goals and respects cultural heritage.

In conclusion, Australian native grains offer an opportunity to reshape food systems through a more diverse, resilient, and culturally inclusive grain sector. Realising this vision will require interdisciplinary research, investment in post-harvest infrastructure, robust agronomic trials, and consumer education. Functional characterisation—especially around hydration, foaming, and pasting behaviour—can bridge nutritional potential with practical product development. As interest in sustainable food sources grows globally, native grains may find their place not just as alternatives but as integral parts of a reimagined Australian grain economy.

The functional profiles of the native grains examined in this study suggest potential applications across diverse food systems. The low bulk density of Native Millet and Weeping Grass indicates promise in extruded snack products, where expansion and texture are critical.

The high water and oil absorption capacities of Weeping Grass suggest suitability for gluten-free baked goods and plant-based foods requiring strong hydration and fat-binding. Curly Mitchell Grass, with its relatively high bulk density and notable foaming capacity, may be applicable in aerated bakery products such as cakes and biscuits. Button Grass, with moderate hydration and oil absorption properties, could be used in flatbread or as a partial substitute in blended flour. These applications highlight the scope for integrating native grains into modern food systems while diversifying Australia's grain sector.

In this thesis, 'wholegrain' refers to the use of the entire grain (bran, germ, and endosperm), whereas 'wholemeal flour' refers to flour milled from whole grains without removing any anatomical components.

This PhD project aimed to:

1. Characterise the morphological features of the grains of four Australian native grasses—*Panicum decompositum*, *Microlaena stipoides*, *Astrebla lappacea*, and *Dactyloctenium radulans*—compared to domesticated cereals (Chapter 2). Characterisation included determining grain dimensions, identifying the structural organisation of the embryo, endosperm, and aleurone layer, and mapping the distribution of starch, protein, and β-glucan using bright-field and fluorescence microscopy.
2. Determine the proximate composition (moisture, protein, fat, ash, and total carbohydrate) of the selected native grains, and quantify key functional properties—namely, water absorption, oil absorption, foaming capacity at different pH levels, and bulk density—to assess their potential application in food systems (Chapter 3)
3. Determine the carbohydrate profile of the native grains, including total starch, amylose content, total dietary fibre, resistant starch, and β-glucan (Chapter 4).
4. Profile the protein content and composition of the native grains through Osborne fractionation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with barley serving as a control (Chapter 5)
5. Characterise the proteomic profiles of the native grains using LC-MS and compare them to the barley proteome (Chapter 6).

Chapter 2

Morphological properties of Australian native grains

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Indigenous Australian grass seeds as grains: macrostructure, microstructure and histochemistry

Farkhondeh Abedi, Claudia Keitel, Ali Khoddami, Salla Marttila,
Angela L. Pattison and Thomas H. Roberts

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2.1 Introduction

The fruit of grass is a caryopsis, which holds a single seed considered the greater part of the mature fruit. The seed consists of the embryo, endosperm, nucellus, testa (seed coat) and fruit coat (pericarp) firmly bound to the testa (Evers *et al.*, 1999). The embryo consists of an embryonic axis that includes the plumule and radicle, and the scutellum between the embryonic axis and the endosperm. The scutellum transfers nutrients from the endosperm to the embryo (Evers & Millar, 2002).

The endosperm is the major storage tissue of caryopsis and is made up of two sub-tissues: the starchy endosperm and the aleurone. The storage proteins and starch in the starchy endosperm are hydrolysed by enzymes produced in the aleurone layer to nourish the embryo during germination. The aleurone layer cells surround the starchy endosperm and produce the enzymes required for this mobilisation (Antonini *et al.*, 2018; Evers & Millar, 2002).

The identity, proportions and position of the major storage components of the grain and their distribution and interactions at finer scales play an important role in determining the nutritional and processing properties of the grain and flour (Irving & Jideani, 1997). The microstructure of plant-based foods can influence the digestion of dietary macronutrients. Starch, proteins and lipids, for instance, are less susceptible to enzyme attack during digestion when encapsulated inside cell walls (Bhattarai *et al.*, 2018).

The economic cost of grain processing is a key to determining whether a particular species can be profitable in contemporary markets. Morphological traits (e.g., kernel size, shape, and density) play a critical role in the time and energy required during processing (Edwards *et al.*, 2008). Investigating these physicochemical and morphological properties contributes to understanding the opportunities and challenges associated with the commercial production of native grains.

This study examined the microstructure and histochemistry of the grains of four Australian native grasses: *Dactyloctenium radulans* (Button Grass)*, Astrebla lappacea* (Curly Mitchell Grass)*, Panicum decompositum* (Native Millet)*,* and *Microlaena stipoides* (Weeping Grass). These species were selected based on their yield potential in their natural range, threshing simplicity, and potential for commercial food applications.

This study aimed to analyse grain dimensions, localise and image the embryo, endosperm and aleurone layer, and determine the distribution of starch, protein, and beta-glucan in the grains using bright-field and fluorescence microscopy.

The results of this study were expected to link traditional knowledge of food properties of the grains with physical features at a microscopic level, explain and interpret nutritional information associated with these grains, and provide a foundation for future research on specific food applications of these grains.

2.2 Material and Methods

2.2.1 Plant Materials

Australian native grains (mature seeds) selected for this study were from Button Grass (*Dactyloctenium radulans)*, Curly Mitchell Grass (*Astrebla lappacea*), Native Millet (*Panicum decompositum*), and Weeping Grass (*Microlaena stipoides*). Sorghum (*Sorghum bicolor* cv. Buster), barley (*Hordeum vulgare* cv. Spartacus), and wheat (*Triticum aestivum* cv. Kord) grains were used as domesticated comparator grains. Control grains were analysed as commercial grain lots; detailed agronomic conditions (site, season, inputs) were not available. The grains from Button Grass and Native Millet were harvested by hand from around 100 plants in each case, and the seeds were pooled.

Button Grass and Native Millet seeds were collected from the University of Sydney Plant Breeding Institute and Llara farm, both located in Narrabri (latitude: -30° 18’ 60” S; longitude: 149° 45’ 60” E) in northern NSW, Australia. Curly Mitchell Grass seeds were obtained from Thallon (latitude: -28° 38’ 60” S; longitude: 148° 51’ 60” E), Queensland, and Weeping Grass seeds were supplied by Creative Native Food Service Co, Australia, harvested from a farm outside of Armidale (latitude: -30° 30’ 30” S; longitude: 151° 40’ 16” E), NSW in May 2021. Seeds were threshed with methods best suited to each species (Pattison, McGee*, et al.*, 2023), cleaned manually by sifting and winnowing to remove impurities such as chaff and dust, and maintained at 4˚C in sealed containers until analysis.

2.2.2 Grain Shape, Size, and Weight

For external morphology analysis, images of the whole grains were taken using a Leica MZ16FA fluorescence stereomicroscope (Australian Centre for Microscopy and Microanalysis, University of Sydney). Microscopy images of 20 grains of each native species were used to determine grain lengths and widths using Fiji ImageJ ver1.54c. The weights of 20 seeds of each species were measured using an analytical balance with a readability of five decimal places.

2.2.3 Grain microstructure and histochemistry

2.2.3.1 Paraffin Embedding

Sample preparation

The grains were fixed in a 10% formaldehyde solution overnight, washed in distilled water (3 × 30 min) and dehydrated with an increasing ethanol series: 25%, 50%, 75%, and 95% (20 min each), then with 100% ethanol (3 × 30 min). Samples were then incubated in chloroform—as a clearing agent—overnight and immersed in melted paraffin wax at 60˚C (72 h). Grains were then embedded in paraffin blocks.

Longitudinal sections (5 µm thick) were prepared with a Leica EM UC7 ultramicrotome using a Diatome histo diamond knife and collected on microscope slides. Sections were deparaffinised and rehydrated (Table 2.1).

Table 2.1 Deparaffinising and rehydrating protocol

Step	Solution	Time (min)
1	Xylene	5
2	Xylene	5
3	Xylene	5
4	100% ethanol	5
5	100% ethanol	5
6	100% ethanol	5
7	70% ethanol	5
8	Running tap water	1

Sample Staining

For bright-field microscopy, sections were stained with 0.2% Light Green, which stains proteins green, followed by ready-to-use liquid Lugol's iodine, which stains the starch granules blue to black (2 min each). For fluorescence microscopy, sections were stained with 0.1% acid fuchsin for 4 min, which stains protein red, followed by 0.01% calcofluor white for 1 min, which stains cell walls blue if they contain β-glucan or cellulose (Jääskeläinen *et al.*, 2013; Nicholas *et al.*, 1994).

Preparation of Stains

A. 0.1% Acid fuchsin: 0.1 g acid fuchsin + 100 ml Milli Q water + 100 μl glacial acetic acid

B. 0.01% Calcofluor white: liquid calcofluor white + 1 drop of NaOH or KOH

C. 0.2% Light Green: 0.2 g Light Green + 100 ml distilled water + 0.2 ml glacial acetic acid

D. Ready-to-use liquid Lugol's iodine solution

Microscopy

After each staining, sections were washed under running tap water for 1 min and dried at room temperature. The sections were mounted with one drop of glycerol, a coverslip added, and the samples were imaged using an Olympus VS120 slide scanner at 40× magnification.

2.2.3.2 Resin Embedding

Sample preparation

Grains were fixed in a 4% formaldehyde solution at 4°C for 24 h, sectioned longitudinally to 2–3 mm thickness, and fixed again overnight. The sections were washed in distilled water (3 × 30 min) and dehydrated with an increasing ethanol series: 30%, 50%, 70%, and 90% (30 min each), then with 100% ethanol (3 × 30 min). Samples were then infiltrated with LR White resin at 25%, 50%, 75% (12 h each) and 100% (2 ×12 h). Samples were then positioned in Beem embedding capsules, filled with 100% LR White resin to the top, and polymerised at 60 °C (48 h) (Zhao *et al.*, 2016).

Longitudinal sections (1 μm thick) were prepared using a Leica EM UC7 ultramicrotome with a Diatome histo diamond knife and collected on microscope slides.

Sample Staining

Since Light Green and Lugol's iodine stains did not penetrate the resin-embedded sections (resin was not removed before staining)—unlike in paraffin embedding—methylene blue was selected as an alternative stain. Thus, for bright-field microscopy, sections were stained with 0.1% methylene blue for 2 min to observe cell walls (blue) and starch (white). For fluorescence microscopy, sections were stained with 0.1% acid fuchsin for 4 min, which stains protein red, followed by staining with 0.01% calcofluor white for 1 min, which stains cell walls blue if they contain β-glucan or cellulose (Herburger & Holzinger, 2016; Kamal-Eldin *et al.*, 2009; Nicholas *et al.*, 1994).

Microscopy

After each staining, sections were washed under running tap water for 1 min and dried at room temperature. The sections were mounted with one drop of glycerol; a coverslip was added and imaged using an Olympus VS120 slide scanner at 40× magnification. The images selected for the figures presented here were based on the intactness of the sectioned tissue, a lack of shrinking or other damage, and consistent staining over the whole section.

2.2.4 Starch Distribution

Grains were cut transversely using a scalpel. The sections were suspended in 25% Lugol's iodine solution (30 s) at room temperature and observed under a Leica M125 C stereo microscope. The starch stained with Lugol's iodine turned dark blue to black immediately, as expected (Zhao *et al.*, 2022).

2.2.5 Visualisation of the Embryo

The embryo of each of the native grains was visualised using vital staining. Grains were cut longitudinally through the embryo with a scalpel and then immersed in 1% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) solution and placed in the dark for 2 h at room temperature. Microscopy images were taken using a Leica M125 C stereo microscope. Viable embryos turned orange to bright red (Vujanovic *et al.*, 2000).

2.3 Results and Discussion

2.3.1 Whole-grain Shape, Size, Weight, and Colour

The shape, size, weight, and colour of the whole grains are imaged from four angles to highlight the different structures that varied between the four native grain species (Figs. 2.1 and 2.2). The smallest and largest (also heaviest) grains among the species were those of Button Grass and Weeping Grass, respectively, the latter being highly elongated (Fig. 2.1), like that of Kangaroo Grass (Cowley *et al.*, 2023).

Button Grass grains had an ovoid shape, whereas Native Millet grains tended to be long and ellipsoid. Curly Mitchell Grass and Weeping Grass grains were found to be pyriform and canoe-shaped, respectively (Fig. 2.2). The colour of the seeds varied from yellow brown in Curly Mitchell Grass, orange-brown in Button Grass and dark brown in Weeping Grass to shiny dark brown or black in Native Millet (Fig. 2.2).

The shiny appearance of the Native Millet was due to the retention of the lemma and palea, despite applying the most effective threshing method. Fig. 2.2C reveals how tightly and far the lemma is wrapped around the palea, and thus, why these structures are so difficult to remove. Historically, it was common for Indigenous people to incorporate the lemma and palea of Native Millet grain into the final flour (Latz, 1995). In oat grains, this results in an increase in the fibre and a decrease in the digestible carbohydrate content (Ganssmann & Vorwerck, 1995). The Cooper Creek people of Central Australia used two stones—a large, uneven slab and a smaller, ball-shaped one—to grind the whole seeds of Native Millet into a meal, as reported by Gregory in 1887. The seeds were placed on the larger stone and then ground into a meal, sometimes using water to aid in the process (Tindale, 1977; Clarke, 2011).

Native Millet seeds can be ground, with the resulting flour absorbing water (i.e. caking) readily. However, grinding Native Millet seeds is more difficult than for seeds of other species that are both soft and readily fractured (Mildwaters & Clarkson, 2020). In a study by Jenifer et al. (2023), Native Millet wholemeal flour had a poorer ability to form a paste with water, which had a lower viscosity than wheat cv. Spitfire wholemeal flour. This was attributed to the relatively lower starch and higher fibre content found in Native Millet seeds, which were threshed to a similar extent as in traditional methods (Jenifer *et al.*, 2023).

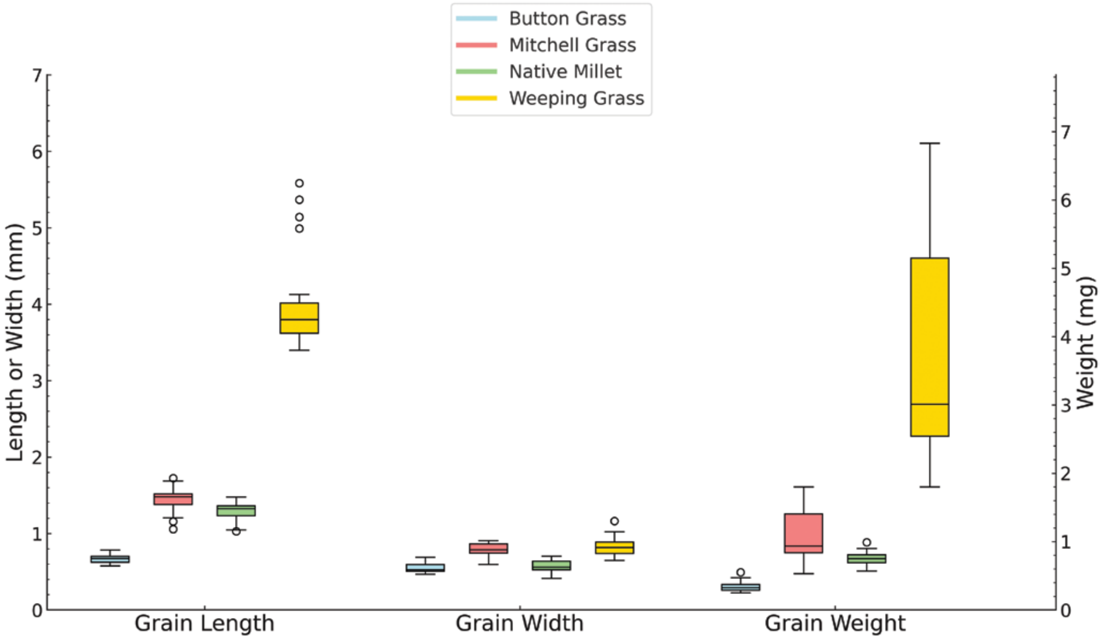

Figure 2.1. Box plot analysis of grain length, width, and weight of the four native grasses (n=20). The length and width (in mm) of the grains were determined using a Leica MZ16FA stereomicroscope and FIJI ImageJ software. The weight of the grains was measured using an analytical five-decimal-place balance. Each box plot represents the distribution of values for Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass, respectively. The horizontal line within each box indicates the median value, while the upper and lower borders show the interquartile range (25th to 75th percentiles). Whiskers extend to the 5th and 95th percentiles. Points outside the whiskers are outliers.

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Figure 2.2. External appearance of the native grains. Images of the whole grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, and (D) Weeping Grass from different angles using a Leica MZ16FA stereomicroscope (scale bar = 100 µm). Note the shiny and stripey palea and lemma surrounding the Native Millet grain.

2.3.2 Endosperm Starch

Most metabolisable energy stored in the endosperm is in the form of starch, non-starch polysaccharides and protein. Starch is stored in the form of granules, the properties of which (such as size distribution) have effects on digestibility and nutrition. Variability in functional characteristics of the starch granules, including water absorption, swelling, pasting and gelling behaviour, as well as susceptibility to enzyme attack, is affected by their natural polydispersity (Wang & Copeland, 2013).

In Button Grass, starch granules were distributed over most of the starchy endosperm but did not extend to the aleurone layer (Fig. 2.3A), whereas in Weeping Grass, starch granules appeared to be distributed out to the aleurone layer (Fig. 2.3D). In Native Millet (Fig. 2.3C), the starch granules appear to be densely packed in the starchy endosperm, as those in wheat (Fig. 2.3F). In Curly Mitchell Grass (Fig. 2.3B), the starch granules appeared to be more concentrated on one side of the grain, whereas in sorghum, the starch was concentrated in the centre of the grain (Fig. 2.3E).

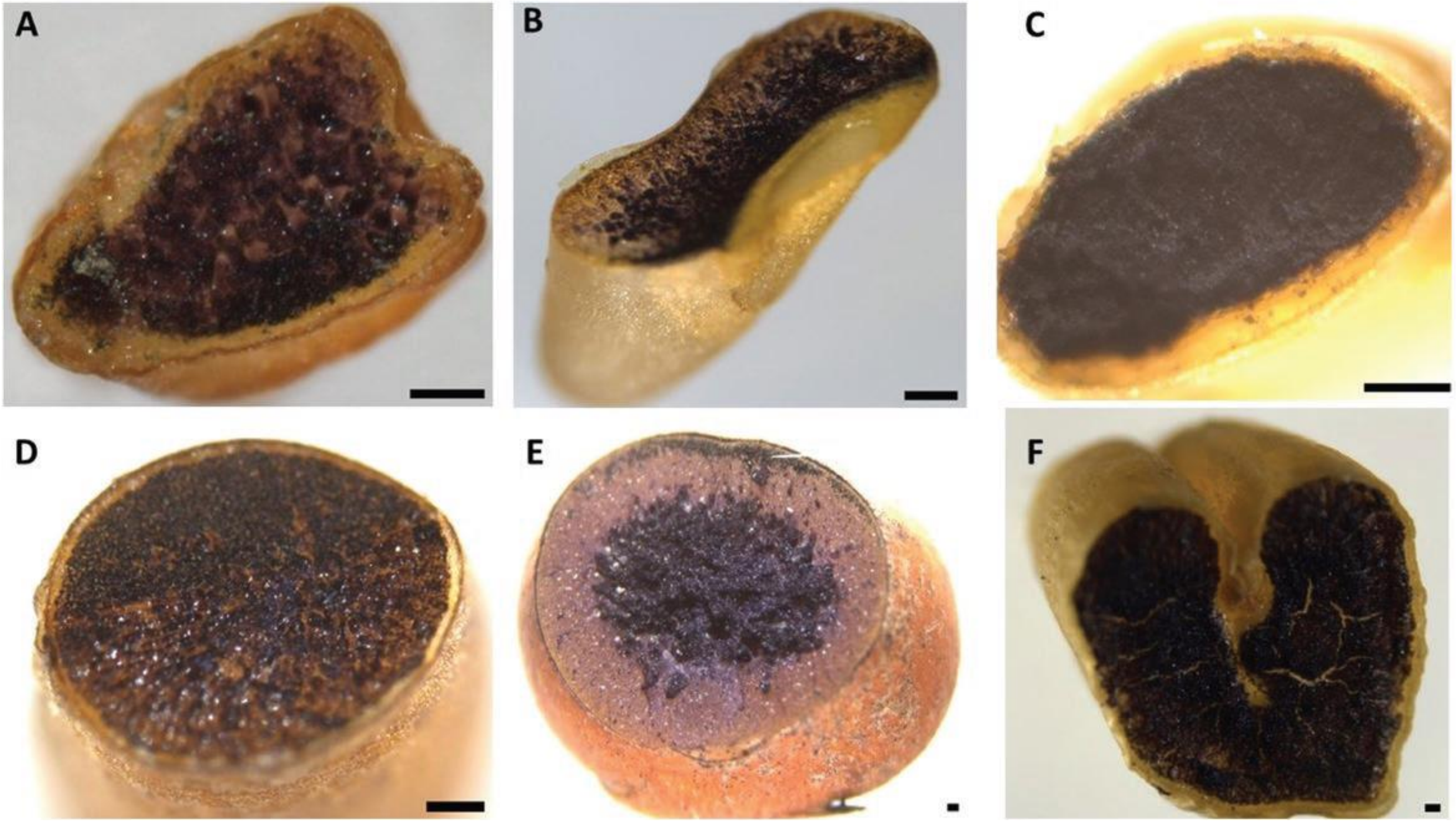

Figure 2.3. Starch distribution in the native and domesticated grains. Grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, (D) Weeping Grass, (E) sorghum and (F) wheat were cross sectioned using a scalpel and infused with 25% Lugol's iodine for 30 s at room temperature, staining the starch black. The grains were observed using a Leica M125 C stereo microscope (scale bar = 100 µm).

2.3.3 Cell Wall Components and Protein

2.3.3.1 Paraffin-Embedded Sections

Paraffin embedding significantly damaged the grain sections, with intact seeds often not retained and many parts lost during the process (Figs. 2.4 and 2.5). Therefore, resin embedding was employed as a more suitable method to preserve the structural integrity of the samples.

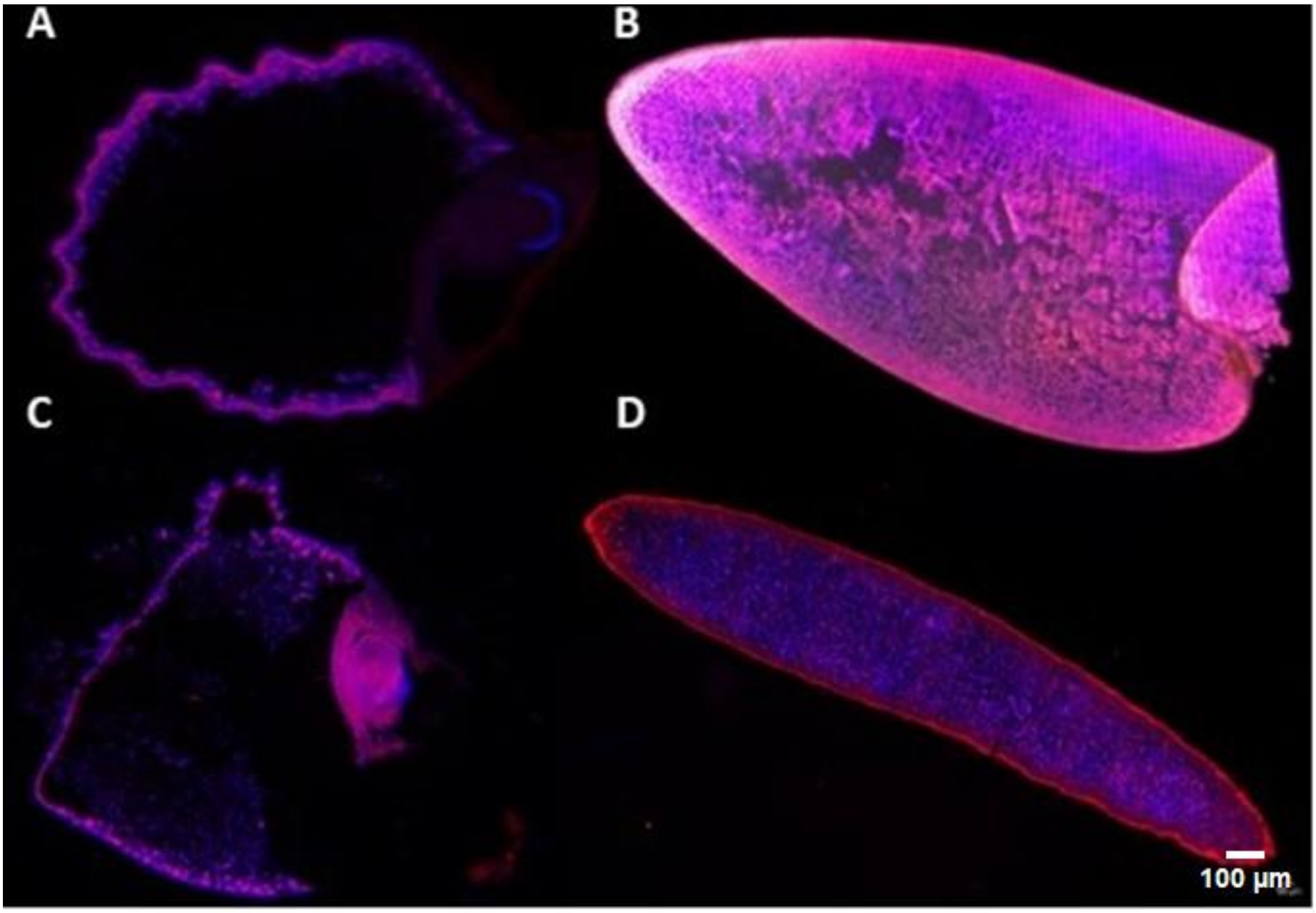

Figure 2.4. Protein and cell wall visualisation of the native and wheat grains. Paraffin-embedded grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, and (D) Weeping Grass were sectioned longitudinally (5 µm) with an ultramicrotome and stained with 0.1% acid fuchsin for 4 min—stains protein red—followed by staining with 0.01% calcofluor white for 1 min—stains cell walls blue if they contain β-glucan or cellulose. The microscopy images were observed using an Olympus VS120 slide scanner at 40× magnification.

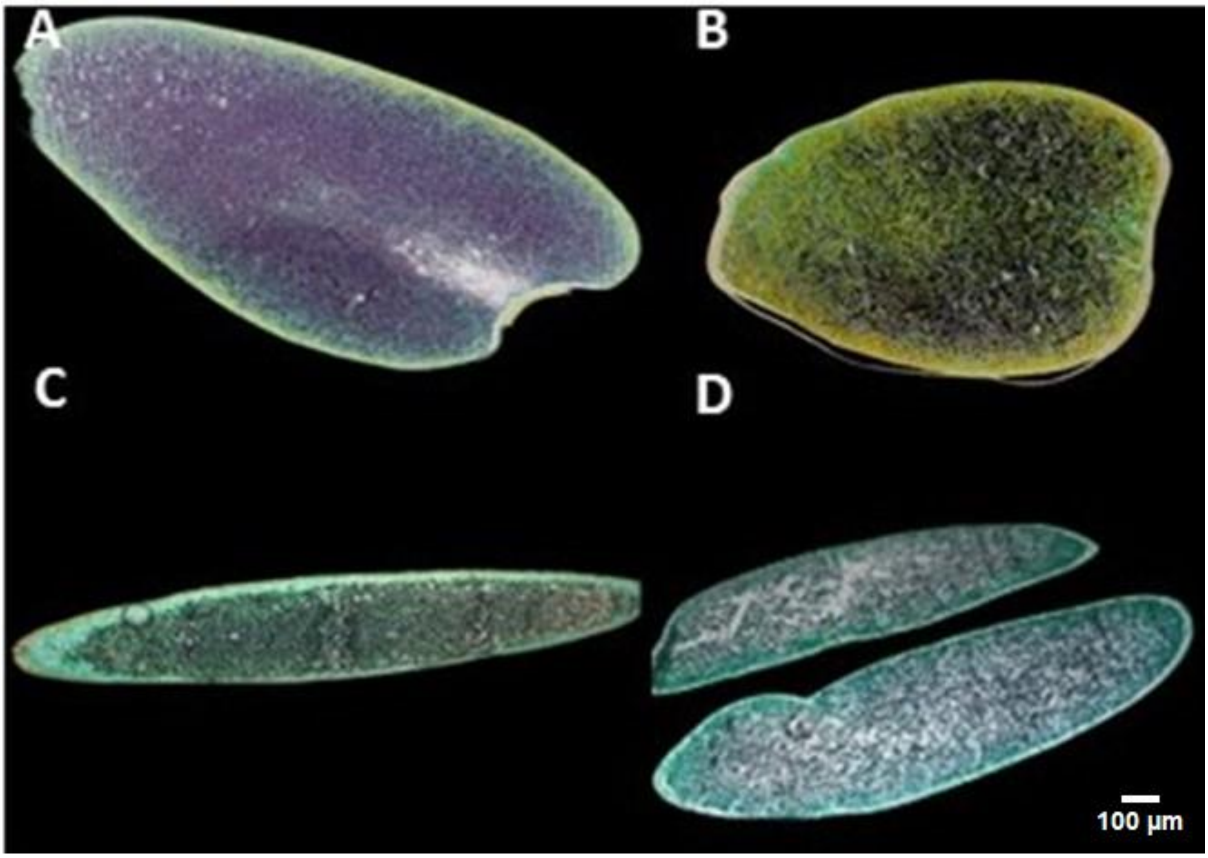

Figure 2.5. Protein and cell wall visualisation of the native and wheat grains. Paraffin-embedded grains of (A and B) Curly Mitchell Grass, (C) Weeping Grass, and (D) wheat were sectioned longitudinally (5 µm) with an ultramicrotome and stained with 0.2% Light Green—stains proteins green—followed by ready-to-use liquid Lugo's iodine—stains starch granules blue to black—for 2 min. The microscopy images were observed using an Olympus VS120 slide scanner at 40× magnification.

2.3.3.2 Resin-Embedded Sections

Observation of resin-embedded and sectioned grains of Button Grass, Curly Mitchell Grass and Native Millet revealed that starch granules were located in the starchy endosperm, with fewer granules in the sub-aleurone layer (Figs 2.6–2.8), as appears to be the case in Kangaroo Grass (Cowley *et al.*, 2023). In contrast, starch granules in Weeping Grass were abundant in the sub-aleurone. In Button Grass, Native Millet and Weeping Grass, the size of the granules appeared to be relatively constant in the starchy endosperm, as in wheat grain. In contrast, the granules in Curly Mitchell Grass were smaller in the sub-aleurone layer compared to those in the central regions of the starchy endosperm, as in sorghum grain. Estimates of typical starch granule size with reference to the 50-µm scale bar are Button Grass 5 µm, Curly Mitchell Grass 5 µm, Native Millet 2–3 µm and Weeping Grass 10 µm (Fig. 2.8).

In all the native species, the starchy endosperm cell walls were thinner than in the domesticated grains. Weeping Grass appeared to have the thickest endosperm cell walls among the native species (Figs 2.6–2.8), which is in line with an earlier finding that weeping grass retained entire endosperm cell wall structure at maturity, as opposed to the remnants of cell walls retained in most cereals (Shapter *et al.*, 2008). In Curly Mitchell Grass, Native Millet and Weeping Grass, the endosperm cell walls were strongly stained blue by calcofluor white, as in Kangaroo Grass (Cowley *et al.*, 2023), β-glucan is found mainly in the endosperm of cereal grains and is categorised nutritionally as a soluble dietary fibre component, whereas cellulose is insoluble. Barley and oats are well-known to be high in β-glucan, but the amount of β-glucan present differs in both these species depending on the genotype (up to 15% by weight for barley and up to 7% by weight for oats). Typically, β-glucan is evenly spread throughout the endosperm of barley grains, while it is more concentrated in the outer layers of oat grain endosperm (Autio & Salmenkallio-Marttila, 2001; Vasanthan & Temelli, 2008).

Several nutritional studies have established a correlation between regular consumption of foods containing cereal β-glucan in appropriate concentrations and a decreased likelihood of chronic health issues. By reducing blood serum cholesterol levels, β-glucan helps mitigate the risks associated with cardiovascular disease (Braaten *et al.*,1994). Additionally, it aids in regulating blood glucose levels, thereby contributing to the management of diabetes (Wood *et al.*, 1994).

Although grains of grasses generally have low cellulose contents (Fincher & Stone, 2004), and thus high β-glucan in the endosperm is the more likely explanation for the observations in Fig. 2.7, there is an interesting exception in rice, where endosperm cell walls of mature rice grains contain significant amounts (up to 30%) of cellulose (Shibuya & Nakane, 1984).

Based on the observed red or pink staining in sections stained with acid fuchsin (Fig. 2.7), endosperm protein was most concentrated in the aleurone and sub-aleurone layers of all the native grains, except Button Grass, in which the starchy endosperm appeared to be rich in protein, as in wheat and sorghum grain. In Button Grass, the endosperm cell walls were stained with a mixture of blue and pink, suggesting the presence of both protein and β-glucan (Fig. 2.7A). The red stain of the aleurone cells in all species indicated an abundance of protein in that tissue (Fig. 2.7).

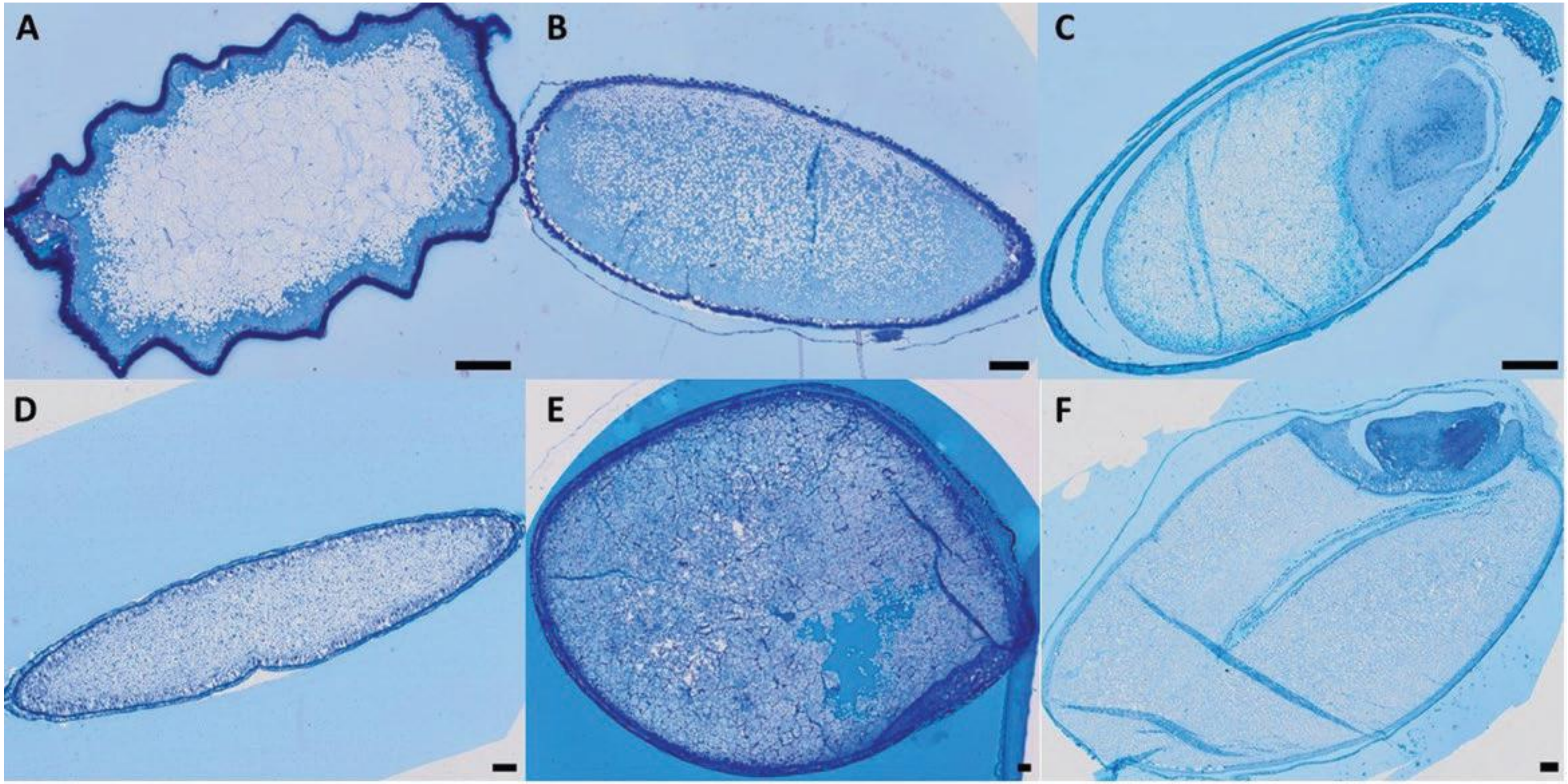

Figure 2.6. Tissue structure of the native and domesticated grains. Resin-embedded grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, (D) Weeping Grass, (E) sorghum and (F) wheat were sectioned longitudinally (1 µm) with an ultramicrotome and stained with 0.1% methylene blue for 2 min. The tissue structure (stained) was observed using an Olympus VS120 slide scanner at 40× magnification (scale bar = 100 µm). Starch appears white, whereas cell walls (specifically beta-glucan and cellulose) appear stained. Note the palea and lemma surrounding the Native Millet seed (C).

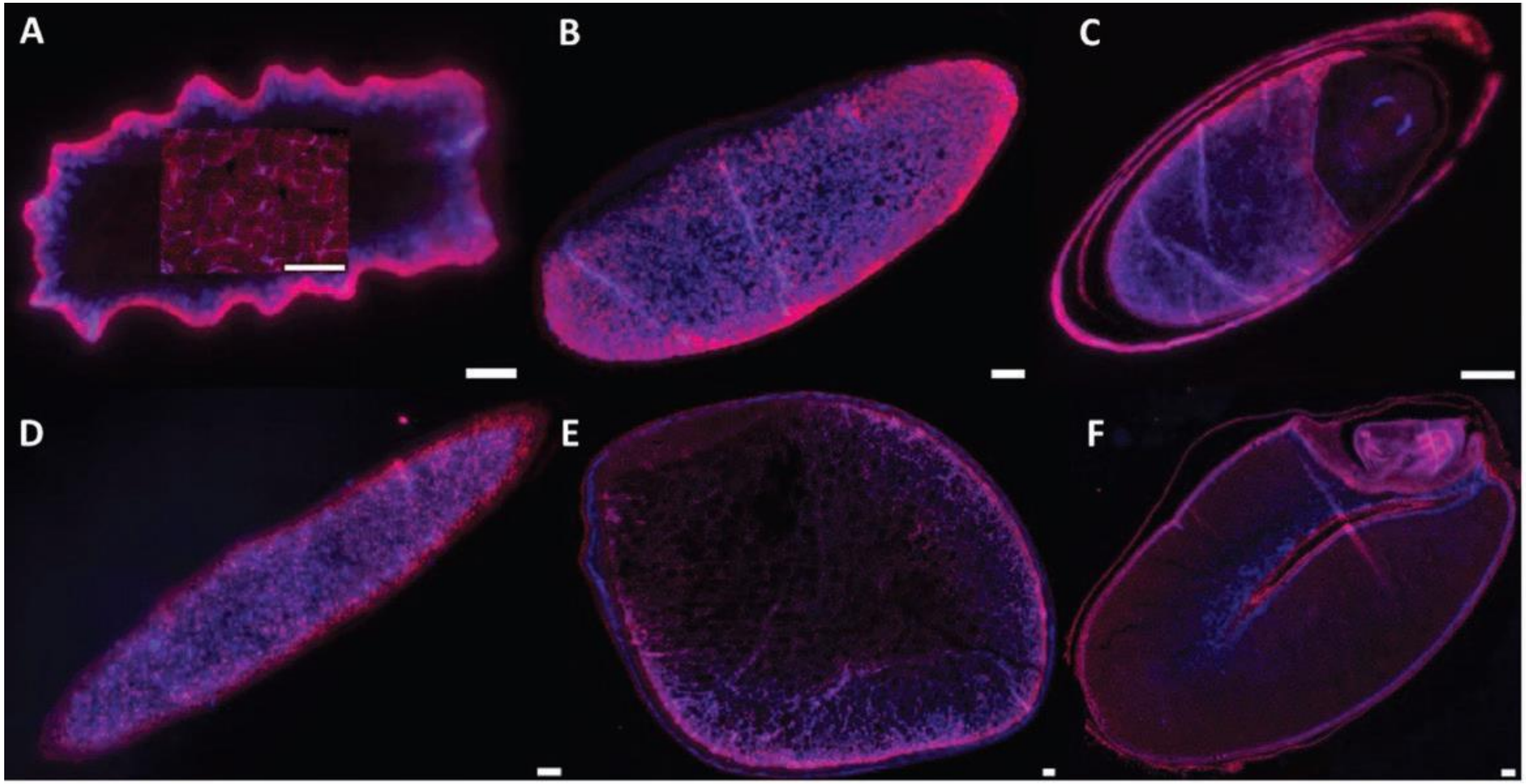

Figure 2.7. Protein and cell wall visualisation of the native and domesticated grains. Resin-embedded grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, (D) Weeping Grass, (E) sorghum and (F) wheat were sectioned longitudinally (1 µm) with an ultramicrotome and stained with 0.1% acid fuchsin for 4 min and 0.01% calcofluor white for 1 min. The staining showing proteins in red and cell walls (containing β-glucan or cellulose) blue, respectively, was observed using an Olympus VS120 slide scanner at 40× magnification (scale bar = 100 µm). The section in the box in the middle of the Button Grass image shows the endosperm structure at a higher exposure setting but at the same magnification. Note the palea and lemma surrounding the Native Millet seed (C).

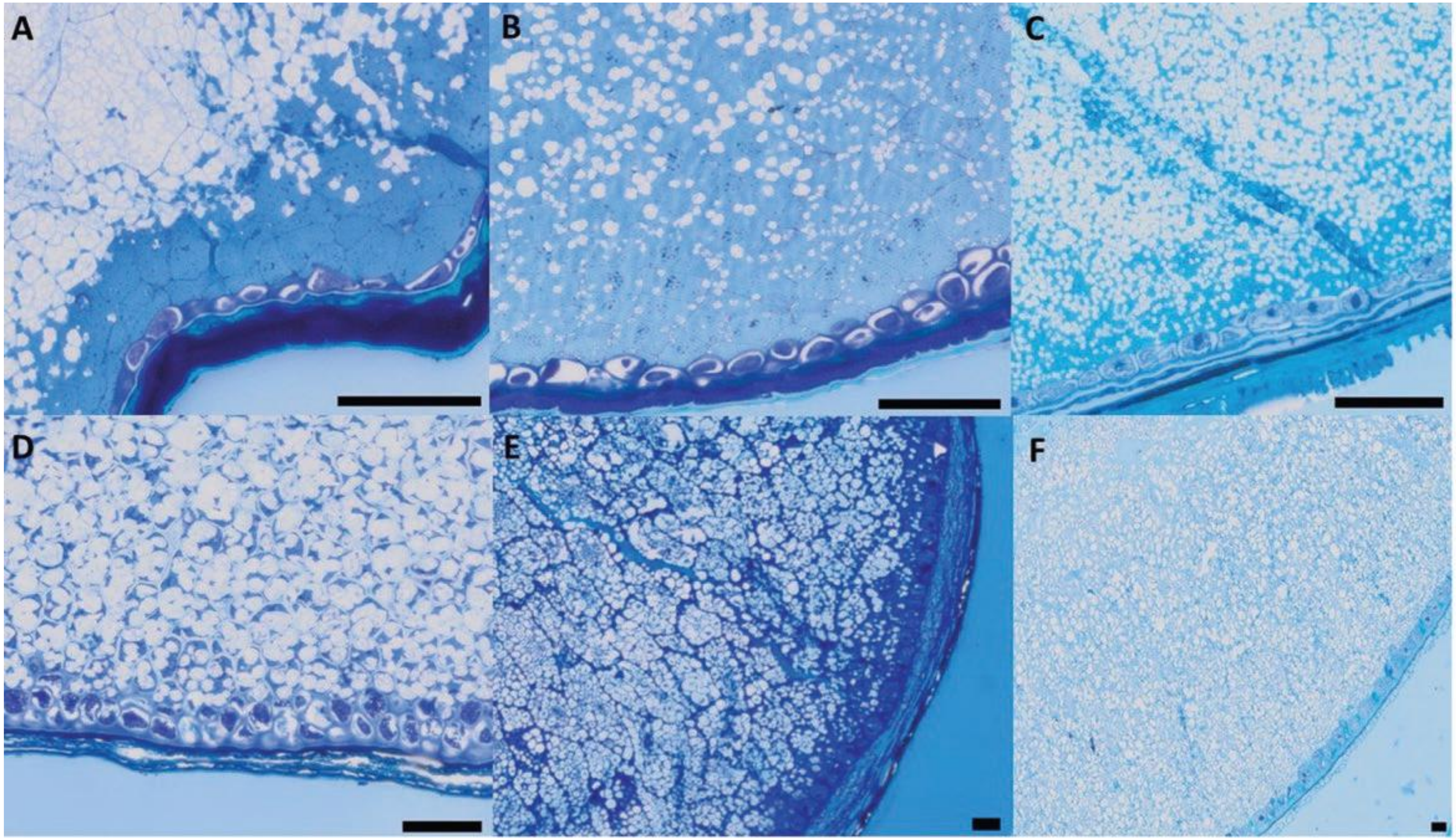

Figure 2.8. Detailed structure of the aleurone layer and starchy endosperm of the native and domesticated grains. Close-up microscopy images (based on images from Fig. 2.4) of longitudinal sections (1 µm) of the resin-embedded grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, (D) Weeping Grass, (E) sorghum and (F) wheat, stained with 0.1 % methylene blue for 2 min. The staining showing the aleurone and endosperm cell structure of the grains was visualised using an Olympus VS120 slide scanner at 40× magnification (scale bar = 50 µm).

2.3.4 Aleurone Layer Microstructure

Cells in the aleurone layer produce enzymes required for the mobilisation of the endosperm during seed germination (Evers & Millar, 2002; Antonini *et al.*, 2018) and have significant implications for the nutritional properties and shelf life of the flour. The wheat aleurone layer is highly nutritious as it contains abundant dietary fibre as well as several classes of biologically active compounds, including phenolic antioxidants, phytate, lipids and vitamins B and E. Furthermore, the dietary fibre present in the wheat aleurone layer is classified as a non-starch polysaccharide, playing a significant role in influencing the properties of starch-based foods, such as their rheology and texture. Nevertheless, the oxidative rancidity of wheat flour containing substantial aleurone tissue poses a significant challenge during storage and production, resulting in a shortened shelf life (Jin *et al.*, 2021).

The thickness of the aleurone layer (i.e. the number of adjacent cells), as well as the size and shape of the aleurone cells, varied among the native species (Fig. 2.8). The aleurone layer was 1–2 cells thick in Weeping Grass (Fig. 2.8) (Kasem *et al.*, 2011), but only one cell thick in the other native species, as in Kangaroo Grass (Cowley *et al.*, 2023), as well as in wheat and sorghum grain (Fig. 2.8). In barley, the aleurone is 2–4 cells thick (Paleg & Hyde, 1964; Clutterbuck & Briggs, 1973). The aleurone cells in Weeping Grass appeared to be larger compared to those of the other native species and domesticated grains (Fig. 2.8D). The corresponding cells in Native Millet (Fig. 2.8C) were more block-shaped, like those in sorghum (Fig. 2.8E) and wheat (Fig. 2.8F), compared to the other native species (Fig. 2.8A, B and D).

2.3.5 Embryo Size and Position

With sections of the native grains stained with methylene blue or acid fuchsin/calcofluor white (Figs 2.6C and 2.7C), the embryo—including the plumule, radicle and scutellum—was observed only in Native Millet, which was estimated to comprise almost 40% of the caryopsis, a larger percentage than that for wheat (Figs 2.6F and 2.7F).

Based on TTC staining of longitudinally cut whole grains, the embryo of Button Grass was located closer to the edge of the grain than the embryos of the other species (including sorghum and wheat) and orientated in parallel to the edge; it was also at the top of the long edge rather than central to the shorter edge (Fig. 2.9A). The embryos of the other species were at the pole of the grain (Fig. 2.9 B, C, E and F). In Weeping Grass, the embryo did not stain (~10 seeds were tested), suggesting that the seed may have been unviable (Fig. 2.9D), but further viability testing will be required to clarify this tentative conclusion.

Curly Mitchell Grass appeared to have the largest embryo among the native and domesticated species, constituting almost half of the grain (Fig. 2.9B). The health benefits of consuming foods made from grains of *Astrebla* spp. were well known to Gamilaroi and Yuwaaliyaay people, and this knowledge was maintained within their language (Pattison *et al.*, 2023). The large embryo relative to the size of the whole grain of Curly Mitchell Grass (Fig. 2.9B) may make the flour relatively susceptible to rancidity (as embryos have a high lipid content), like in pearl millet flour, which also contains a large embryo (Nantanga *et al.*, 2008).

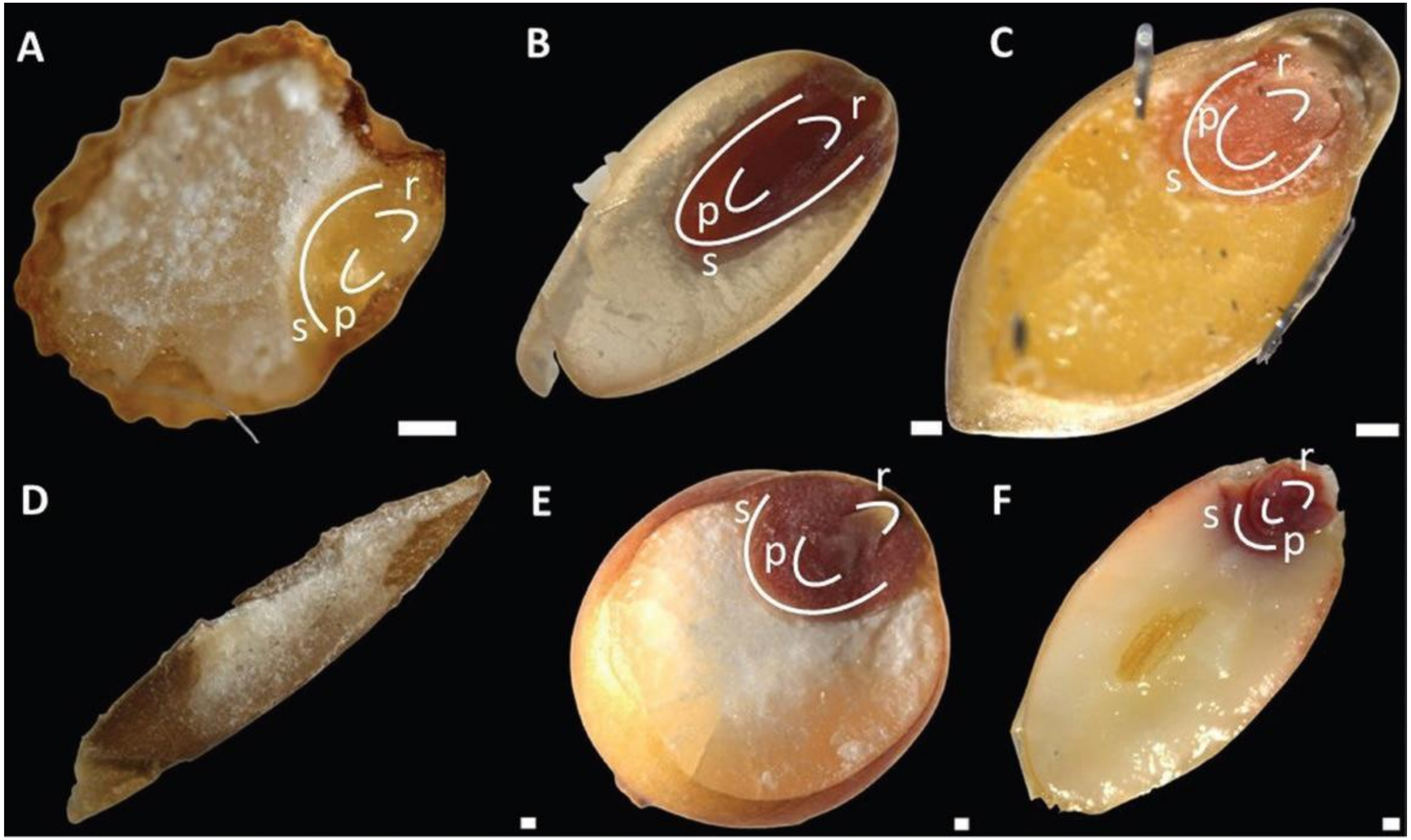

Figure 2.9. Visualisation of the embryo in native and domesticated grains. Grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, (D) Weeping Grass, (E) sorghum and (F) wheat were cut longitudinally through the embryo using a scalpel and stained with 1% (w/v) TTC solution in the dark for 2 h at room temperature. The grains were observed with a Leica M125 C stereo microscope, showing the embryo structure, size, and location. TTC stained the viable embryos orange to bright red. The images illustrate the radicle (r), plumule (p), and scutellum (s) (scale bar = 100 µm). The location of these structures in the Weeping Grass grains could not be determined.

2.4 Conclusion

The utilisation of grains of local grasses by Australia's First Nations people for food and connection to country has largely been lost due to colonisation. Native Australian grain production can potentially deliver environmental, economic, nutritional, and cultural benefits to First Nations people and the wider community. Revitalisation of the native grain food system can only be achieved if relevant properties of the grains are elucidated.

The macrostructure, microstructure, and histochemistry of the native grains examined in this study build on previous research into their nutritional composition and establish a foundation for further investigations into their food applications. By complementing chemical analysis, these microscopy data have aided in comprehending and visually representing structural differences in the grains of Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass, all native grasses of great importance to many First Nations peoples, including the Gamilaroi people of northern NSW.

This type of analysis is applicable to other native grains in Australia (Cowley *et al.*, 2023) and in other countries. The microscopy analysis of the four selected Australian native grain species and the localisation of specific macronutrients within the caryopsis should stimulate further research into the connections between the structural, processing, and nutritional properties of these grains. Specific considerations include the size and location of the embryo, the distribution of starch, β-glucan/cellulose, protein, and the thickness of the aleurone layer.

Chapter 3

Proximate analysis and functional properties of Australian native grains

This chapter is an extended version of part of a revised manuscript submitted to

Applied Food Research

Farkhondeh Abedi, Claudia Keitel, Ali Khoddami, Angela L. Pattison and Thomas H. Roberts

3.1 Introduction

This chapter focuses on the proximate composition and functional analysis of the four native grains studied in this PhD project—*Dactyloctenium radulans* (Button Grass), *Astrebla lappacea* (Curly Mitchell Grass), *Panicum decompositum* (Native Millet), and *Microlaena stipoides* (Weeping Grass), with wheat, barley, and sorghum as comparator grains.

Proximate analysis is a critical method to determine the fundamental nutritional components of food materials—moisture, protein, lipid, ash, and carbohydrate content—and provides insights into grain properties during processing and potential applications in diverse food systems.

Water is a critical component of cereal grains and grain-based products and affects their quality and processing. Moisture content—commonly defined as the mass of water per unit mass of the grain—influences key factors such as harvest suitability, handling, storage stability, and end-use properties. Higher moisture levels at harvest are often associated with increased kernel damage and accelerated deterioration, primarily due to mould growth. As water does not provide nutritional value, sellers and buyers closely monitor it since market standards often impose maximum moisture limits. In consequence, moisture content not only impacts the physical, chemical, and biological properties of cereal grains but also plays an important role in determining their market value (Sadaka & Rosentrater, 2022).

The average protein content of most cereal grains ranges from 8% to 11% on a dry-weight basis (Koehler & Wieser, 2013). In wheat, most of the total grain protein—approximately 80–85%—is in the starchy endosperm, which constitutes the largest portion of the kernel. Although the endosperm primarily comprises starch, it contains around 13% protein by dry weight. The embryo, despite representing a smaller fraction of the grain, is notably richer in protein, typically comprising 25–35% of its dry weight. The bran, including the aleurone layer, also exhibits a high protein concentration of approximately 30%. Genotype and environmental factors, such as soil quality, climate, and nitrogen fertilisation, notably influence both the total protein content and its distribution among grain tissues (Brandolini & Hidalgo, 2012; Khalid *et al.*, 2023; Lebert *et al.*, 2022).

In cereal grains, lipids are distributed across different tissues and occur in distinct forms. For example, lipid is primarily stored in the embryo as triacylglycerols (TAGs)—esters of fatty acids and glycerol—even though the embryo comprises only a small portion of the grain. In wheat and barley, such lipids account for approximately 2–3% of the seed's dry weight, with linoleic acid (C18:2), palmitic acid (C16:0), and oleic acid (C18:1) being the main fatty acids accumulated (Barthole *et al.*, 2012). Beyond the embryo, cereal lipids are mainly located in the bran, or endosperm, depending on the species. Moreover, lipids associated with starch can be classified as either surface or internal lipids based on their extractability with *n*-propanol. Surface lipids, which coat the exterior of starch granules, have a composition similar to that of the bulk kernel lipids (Wang *et al.*, 2020). In contrast, internal lipids—mainly monoacyl lipids (lysophospholipids) and free fatty acids—are incorporated into the starch by forming complexes with amylose. The lipid's hydrocarbon chain in these complexes is enclosed within the hydrophobic amylose helix while the polar end remains exposed. Although these internal lipids constitute only about 1% of the starch, they affect starch swelling, gelatinisation, and ultimately, the baking quality of cereal-based products. In oats, a substantial portion of lipids exists as surface lipids within the starchy endosperm (Moazzami *et al.*, 2011). These surface lipids are believed to consist mainly of monoacyl non-starch lipids that may have migrated into the starch granules during the isolation process. These lipids are predominantly monoacylglycerols, with smaller amounts of diacylglycerols, triacylglycerols, and free fatty acids (Liukkonen & Laakso, 1992).

Wheat flour milling aims to separate the endosperm from the bran and germ as cleanly as possible and then grind the endosperm into flour. Traditionally, flour purity is assessed by its ash content—the inorganic residue left after incineration—which increases from the centre to the outer layers of the wheat kernel. A lower ash content indicates reduced contamination by bran and germ. Although ash content is not considered a reliable predictor of baking quality, its strong correlation with flour colour, ease of measurement, and established market preferences makes it one of the most important indicators of wheat flour quality. Furthermore, ash content is closely linked to the accuracy of bran removal during milling, which influences the flour's functional properties and sensory quality (Kim & Flores, 1999; Li *et al.*, 2023).

Functional properties are the physical and chemical characteristics that affect how cereal grain flours behave during the processing and preparation of food products. They determine the performance of cereal flours in food applications such as baked goods, thickeners, emulsions, doughs, and batters. These properties help select suitable grains for specific food products and improve processing efficiency and consumer acceptance (Awuchi *et al.*, 2019). Functional properties are closely linked to grain composition (e.g., protein, starch, lipid, and fibre content).

Functionality is typically evaluated by measuring parameters such as water absorption capacity (WAC), oil absorption capacity (OAC), swelling capacity, foaming capacity, and bulk density (Modipuram, 2013). These measurements provide valuable insights into the potential uses of cereal flours, particularly when evaluating underutilised species like Australian native grains.

WAC, for instance, is useful in gluten-free doughs and other hydrated products, as it helps retain moisture and improve texture. A moderate OAC can enhance the mouthfeel of plant-based foods by adding richness and supporting flavour delivery. Foaming capacity also plays an important role in baked and aerated products, where it contributes to volume and lightness, although its effectiveness depends on the type and structure of the proteins present. While low bulk density can make packaging and transport more difficult, it is useful in extruded foods because it supports better expansion and texture (Nandane *et al.*, 2025).

The objectives of experiments reported in this chapter were to:

1. Determine the proximate composition (moisture, protein, lipid, ash, and total carbohydrate) of four Australian native grains—Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass—and compare them with wheat, barley, and sorghum as control cereals.
2. Evaluate key functional properties of these grains: water absorption capacity, oil absorption capacity, bulk density, and foaming capacity.
3. Interpret the implications of these compositional and functional traits for the potential application of Australian native grains in food systems.

3.2 Materials and Methods

3.2.1 Plant Materials

The plant materials used in this study were the same as those described in Section 2.2.1 of Chapter 2. Control grains were wheat (*Triticum aestivum* cv. Kord), barley (*Hordeum vulgare* cv. Spartacus), and sorghum (*Sorghum bicolor* cv. Buster), as described in Section 2.2.1. Control grains were analysed as commercial grain lots; detailed agronomic conditions (site, season, inputs) were not available.

3.2.2 Flour Preparation

Whole-grain samples were ground using a Laboratory Mill 3100 (Perten, Australia) with a 0.5-mm screen size before analysis. All tests were conducted in triplicate, except for moisture, total lipid content, and all functional property tests, which were performed in duplicate due to limited sample availability. Whole-grain flour for each species was prepared from the same batch of grains, with each batch derived from multiple plants of the respective species.

3.2.3 Moisture

The moisture content in the native and control grains was measured following the AACC 44–15.02 method (American Association of Cereal Chemists-International, 2000). For this process, 2-g samples were placed into previously weighed aluminium dishes. After being covered and accurately weighed, the dishes were placed without their covers, alongside the lids, into an oven at 135 ± 1°C for 2 h. The timing began once the oven had reheated back to 135°C, which took ~2 min. The dishes were then removed from the oven, immediately covered, and set in a decorator to cool to room temperature (RT) for ~1 h, before their weight was measured. The following formula was used to calculate the moisture content:

Moisture (%) = (A / B) ×100

'A' represents the loss of moisture in grams, while 'B' is the initial weight of the sample in grams.

3.2.4 Total Carbohydrate

Moisture, ash, lipid, and protein percentages were summed and subtracted from 100% to determine the carbohydrate content.

3.2.5 Total Protein

The total protein content of the grain samples was measured using the Dumas combustion method AACC 46-30.01. An Elemental Analyser (Vario CHN Cube, Elementar, Germany) was employed to determine the nitrogen content of the samples, which was then converted to an estimate of total protein.

For nitrogen analysis, samples and standards were accurately weighed into pieces of aluminium foil, folded securely, and loaded into the auto-sampler tray of the Vario CHN Cube elemental analyser. The first five wells were left empty. The next five were allocated for standards, and the remaining wells were used for test samples. In the instrument software, the first two sample positions were set as blanks with a default weight of 1 mg, and the method labeled as “running.” Three additional sample wells were also marked as blanks (1 mg), with the method set as “blank with O_2”.

For the standards, the first two wells contained 40 ± 1 mg of phenylalanine, and the method was set to” running”. The standard material was prepared as follows: a small piece of foil was placed on the balance, the required amount of material was weighed, the foil was folded, and it was placed in the designated well, and the sample order was maintained. The remaining three standard wells also contained 40 ± 1 mg of phenylalanine but were assigned the method “phenylalanine 2.” As part of quality control, one phenylalanine standard was included for every 10 samples using the same method. Test samples were weighed to approximately 100 mg and assigned the method “phenyl 4.” The final sample well was left blank as a control.

Once all samples and standards were loaded, the instrument was operated according to the manufacturer’s instructions.

For the calculation of total protein content, a conversion factor of 5.8 was employed for both the Australian native grains and control samples, as it is considered appropriate for several species of cereal grains, including wheat and barley (Mariotti *et al.*, 2008; Tkachuk, 1969), and accurate conversion factors for the native grains are unknown. Using a consistent factor ensures comparability between different grain types and aligns with widely accepted standards for cereal grain protein assessment.

3.2.6 Total Lipid

The total lipid content of the native and control grain species was measured using Soxhlet extraction, following the principles outlined in the AOAC 920.39C Method for Cereal Fat (Min & Ellefson, 2010). Approximately 2 g of each grain sample was accurately weighed and placed into a Soxhlet thimble. Hexane was added to a pre-weighed, dry round-bottom flask until the solvent level allowed for two siphon cycles. The heated solvent surrounded the sample for 5–10 min before being siphoned back into the boiling flask. This cycle was maintained for 4 h, after which the hexane containing the extracted fat was allowed to evaporate to ensure complete solvent removal. The round-bottom flask, now containing only the extracted fat, was re-weighed. The total fat content of each sample was calculated using the difference in the weight of the pre-weighed flask before and after extraction.

3.2.7 Ash

Ash content was measured as part of the dietary fibre analysis (see 2.5.3). After extracting dietary fibre, the residue was isolated and placed in glass filter crucibles for ash content determination. The ash content, which was subtracted from the total residue weight for the total dietary fibre calculation, was quantified by ashing the samples in a muffle furnace at 550 °C for 5 h. The crucibles containing the residue were placed in a muffle furnace at 550°C for 5 h. After incineration, the crucibles were cooled to RT in a desiccator and then re-weighed. The ash content was calculated by subtracting the initial weight of the crucible and sample from their combined weight after incineration.

3.2.8 Water Absorption Capacity (WAC)

WAC was determined according to Abd Elmoneim *et al.* (2010). A 15-mL centrifuge tube was pre-weighed, and 1.0 g of flour was added. The total weight was then recorded as W_1. After adding 10 mL of distilled water, the tube was vortexed for 1 min. Then, the sample was allowed to stand at room temperature (25 ± 2 °C) for 30 min. The tube was then centrifuged at 4000 × *g* for 25 min, the supernatant decanted, and the tube containing the hydrated flour weighed again (W_2). The following formula was used to calculate WAC:

$$\text{WAC (g/g)} = \frac{(\text{W2} - \text{W1})}{\text{Dry weight sample}}$$

3.2.9 Oil Absorption Capacity (OAC)

The WAC method was adapted to determine OAC, with oil substituted for water. The weight of a 1.0 g sample of flour in a pre-weighed 15-mL centrifuge tube was recorded (W_1), and 10 mL of refined sunflower oil (purchased from Coles Supermarkets, Sydney, Australia) was added to the tube. The mixture was vortexed for 1 min and then allowed to stand at room temperature (25 ± 2 °C) for 30 min. The supernatant oil was gently removed following centrifugation at 4000 × *g* for 25 min. The weight of the tube with absorbed oil was recorded (W_3).

$$\text{OAC (g/g)} = \frac{(\text{W3} - \text{W1})}{\text{Dry weight sample}}$$

3.2.10 Bulk Density (BD)

Bulk density was determined as described previously (Abd Elmoneim & Bernhardt, 2010). A 3.0-g flour sample was carefully placed into a dry, clean 25-mL graduated cylinder to avoid compacting. To settle the contents, the graduated cylinder was tapped 10 times on a laboratory bench from a height of about 5–8 cm. The final volume of flour occupied after tapping was recorded.

Bulk density was calculated using the following formula:

$$\mathrm{BD}\ (\frac{\mathrm{g}}{\mathrm{mL}}) = \frac{\text{Flour weight}}{\text{Final volume after tapping}}$$

3.2.11 Foaming Capacity (FoC)

A 0.75-g sample of flour was accurately weighed and transferred into a clean bottle suitable for homogenisation. Fifteen millilitres of distilled water (V_0) was added to the bottle. The pH of the suspension was adjusted to 4.0, 6.0, 8.0, and 10 using 0.1 M NaOH or 0.1 M HCl, as necessary. The mixture was then homogenised directly within the bottle using a handheld rechargeable milk frother (FITNATE Rechargeable Electric Foam Maker, FITNATE, China) operating at 8,000 rpm for 2 min. Immediately after homogenisation, the entire contents of the bottle, including both foam and liquid, were carefully transferred into a 25-mL graduated cylinder. The foam was allowed to settle for 2 min, after which the total volume occupied by the foam was recorded as $V_{2\ \mathrm{min}}$ (Flores-Jiménez *et al.*, 2024).

Foaming capacity was calculated using the following equation:

$$\mathrm{FoC}\ (\%) = \frac{(\mathrm{V}\ 2\ \mathrm{min} - \mathrm{V0})}{\mathrm{V0}} \times 100$$

3.2.12 Statistical Analysis

For sample characterisation, measurements were performed in triplicate for each flour sample from each species, except for moisture, lipid, and all functional property tests, which were conducted in duplicate due to limited grain availability. All data represent the mean ± standard deviation. Data were analysed using GraphPad Prism v.10.2.1 (GraphPad Software, San Diego, CA, USA). A standard one-way analysis of variance (ANOVA) was conducted to assess differences among the tested groups for all measured parameters. Tukey's *post hoc* multiple comparisons test was performed to identify significant differences between groups. Statistical significance was determined at $p < 0.05$.

3.3 Results and Discussion

The proximate composition of native and control grains is summarised in Table 3.1 and Figure 3.1 to facilitate detailed comparison and visual interpretation. While the table presents exact values, including standard deviations and statistical groupings, the figure provides a comparative overview of values among species.

3.3.1 Moisture

Moisture content, which was determined to allow the other grain components to be expressed on a dry weight basis, was in the range of 10.2–14.7% (Figure and Table 3.1). These values compare well with Australian moisture content receival standards, e.g., 12.5% for milling wheat and malting barley; 13.5% for Australian White Wheat and feed barley (CBH Group ‘Moisture Management’ website).

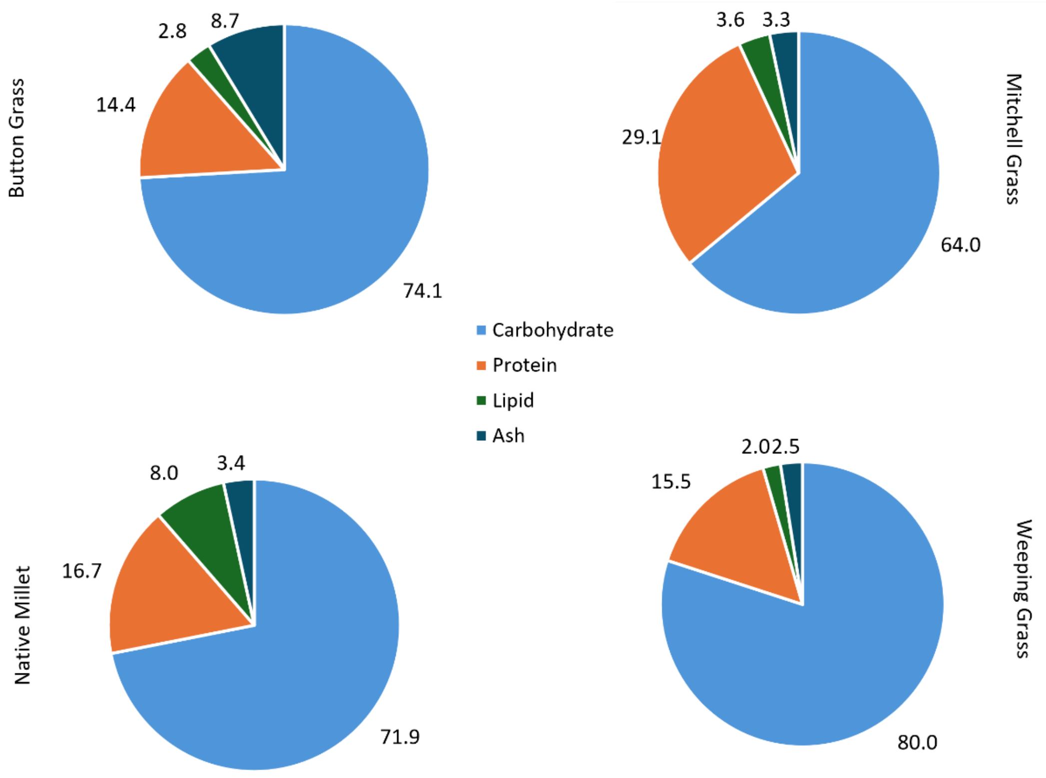

Figure 3.1. Proximate analysis of the native grains—Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass—using wholemeal flour from the same batch. All values are reported on a dry weight basis (g/100 g). Total carbohydrate was calculated by difference. Total protein and ash contents were determined in triplicate (n = 3), and total lipid content was determined in duplicate (n = 2).

Table 3.1. Proximate analysis of native and control grains. The moisture content was reported as a percentage of the total grain weight. Total carbohydrates, protein, lipid, and ash contents were reported on a dry weight basis (g/100 g). The total carbohydrate content was calculated by difference. Data are presented as mean ± standard deviation. Total protein and ash determinations were conducted in triplicate (n=3), while total lipid and moisture determinations were conducted in duplicate (n=2), using wholemeal flour from the same batch of grains. Means with different letters are significantly different ($p < 0.05$), as determined by Tukey's multiple comparisons test following one-way ANOVA.

Species	Moisture	Total carbohydrates	Total protein	Total lipid	Ash
Button Grass	12.3 ± 0.4 $^{b, c}$	74.1 ± 0.4	14.4 ± 0.3 $^{c, d}$	2.8 ± 0.1 ^{d}	8.7 ± 0.3 ^{a}
Curly Mitchell Grass	10.2 ± 0.6 ^{d}	64.0± 0.5	29.1 ± 0.2 ^{a}	3.6 ± 0.4 ^{c}	3.3 ± 0.2 ^{b}
Native Millet	14.7 ± 0.6 ^{a}	71.9 ± 1.0	16.7 ± 0.7 ^{b}	8.0 ± 0.6 ^{a}	3.4 ± 0.4 ^{b}
Weeping Grass	13.5 ± 0.7 $^{a, b}$	80.0 ± 0.4	15.5 ± 0.2 $^{b, c}$	2.0 ± 0.4 ^{e}	2.5 ± 0.1 ^{c}
Wheat	11.3 ± 1.2 $^{c, d}$	82.7 ± 0.4	12.5 ± 0.1 ^{e}	2.5 ± 0.4 $^{d, e}$	2.2 ± 0.2 ^{c}
Barley	13.3 ± 0.6 ^{b}	81.7 ± 0.2	10.7 ± 0.1 ^{f}	4.1 ± 0.1 ^{c}	3.5 ± 0.2 ^{b}
Sorghum	11.0 ±0.1 $^{c, d}$	78.9 ± 1.2	13.2 ± 1.1 $^{d, e}$	5.0 ± 0.0 ^{b}	2.9 ± 0.3 $^{b, c}$

3.3.2 Total Carbohydrate

Among the native grains, the total carbohydrate content was the highest in Weeping Grass and Button Grass (Figure and Table 3.1). Only Weeping Grass had a total carbohydrate content similar to that of the control grains. Curly Mitchell Grass had the lowest carbohydrate content among both the native and control grains (Table 1; $p < 0.05$). Considering its low total carbohydrate content (Figure and Table 3.1), Curly Mitchell Grass might be particularly suitable for low-carbohydrate baked goods, diabetic-friendly foods, and weight management products (Gasparre *et al.*, 2024; Saxelby, 2005).

3.3.3 Total Protein

Total protein contents across the studied native grain species (Figure and Table 3.1) were consistent with those of studies by Brand-Miller *et al.* (1998), Foster *et al.* (2010) and Birch *et al.* (2023). Among the native grains, Curly Mitchell Grass had the highest protein content (29.1 ± 0.2 g/100 g db), roughly double that of Button Grass (14.5 ± 0.3g/100 g db), Native Millet (16.7 ± 0.7 g/100 g db), Weeping Grass (15.5 ± 0.2 g/100 g db), and nearly triple that of barley (10.7 ± 0.1 g/100 g db) ($p < 0.001$).

This result is consistent with the findings of Foster *et al.* (2010), who reported a value of 29.1 g/100 g for the same species. It is also comparable to the 24.68 ± 0.01 g/100 g reported by Williams *et al.* (2024) for the total protein content of an unspecified Australian native grain. The notably high protein content of Curly Mitchell Grass highlights its potential as a nutrient-dense grain, particularly in high-protein or plant-based diets. Such protein levels may also influence its functional performance in food formulations, especially in applications requiring structural or emulsifying properties.

3.3.4 Total Lipid

The lipid content of the native grains exhibited considerable variation between species (Figure and Table 3.1). The values obtained encompass the total lipid content (5.0 ± 0.1 g/100 g) reported by Williams *et al.* (2024) for an unspecified native grain. Native Millet had the highest lipid content with a value of 8.0 ± 0.6 g/100 g db ($p < 0.001$), suggesting its potential as a valuable source of dietary lipids. The variation in lipid content among the native grains highlights the potential for selecting specific grains based on nutritional requirements and dietary goals. For example, Weeping Grass had the lowest lipid content (2.0 ± 0.4 g/100 g) and might hold value in specific dietary applications where lower lipid intake is desired.

The higher lipid content in Native Millet might be due to the apparently large embryo (Abedi *et al.*, 2023). Further research into the specific lipid profiles and fatty acid compositions of these grains would provide deeper insights into their nutritional benefits and potential health impacts.

3.3.5 Ash

Ash content of the native and control grains varied 4-fold between species (Figure and Table 3.1). As ash content is an indicator of the total mineral content, the high ash content in Button Grass may suggest that the grain could be richer in essential minerals such as calcium, magnesium, potassium, and phosphorus (Biel *et al.*, 2021). However, it is also important to consider potential external contamination. As Button Grass grows close to the ground and was harvested from areas near vehicle traffic, the elevated ash content may partly reflect the presence of soil or dust particles deposited on the grain surface.

3.3.6 Water and Oil Absorption Capacity

Among the native species, Weeping Grass exhibited the highest WAC and OAC, which suggests a strong capacity to bind both water and oil (Figure 3.2). The elevated WAC observed for Weeping Grass may be partly related to its compositional characteristics. Weeping Grass was found to contain comparatively high levels of β-glucan and total dietary fibre content (as detailed in Chapter 4), which are known for enhancing water-holding properties (Holtekjølen *et al.*, 2008; Lovegrove *et al.*, 2020). Functionally, high WAC can enhance moisture retention in food systems, which may benefit applications such as bread, meat extenders, or gluten-free products where hydration and textural quality are important (Arora *et al.*, 2023).

Button Grass had a WAC of 1.59 g/g and an OAC of 1.13 g/g, which reflects moderate functionality in hydration and fat-binding applications. Mitchell Grass and Native Millet showed lower WAC values but moderate OAC values. The high bran content in Native Millet may increase the surface area of the flour particles and promote hydrophobic fibre interactions, both of which support oil retention under limited water availability and contribute to its moderate OAC (Elleuch *et al.*, 2011).

Barley's high β-glucan content accounts for its high WAC (Holtekjølen *et al.*, 2008), whereas wheat's abundant gluten accounts for its superior OAC, despite only moderate WAC (Kaushik *et al.*, 2013). In contrast, sorghum had a lower OAC among the control grains, which may be attributed to the nature of its dominant storage protein, kafirin. Kafirin is characterised by a compact, cross-linked structure and low solubility, which limits its surface activity and hydrophobic interactions, thereby reducing its ability to bind oil (Abah *et al.*, 2020; Musigakun & Thongngam, 2007).

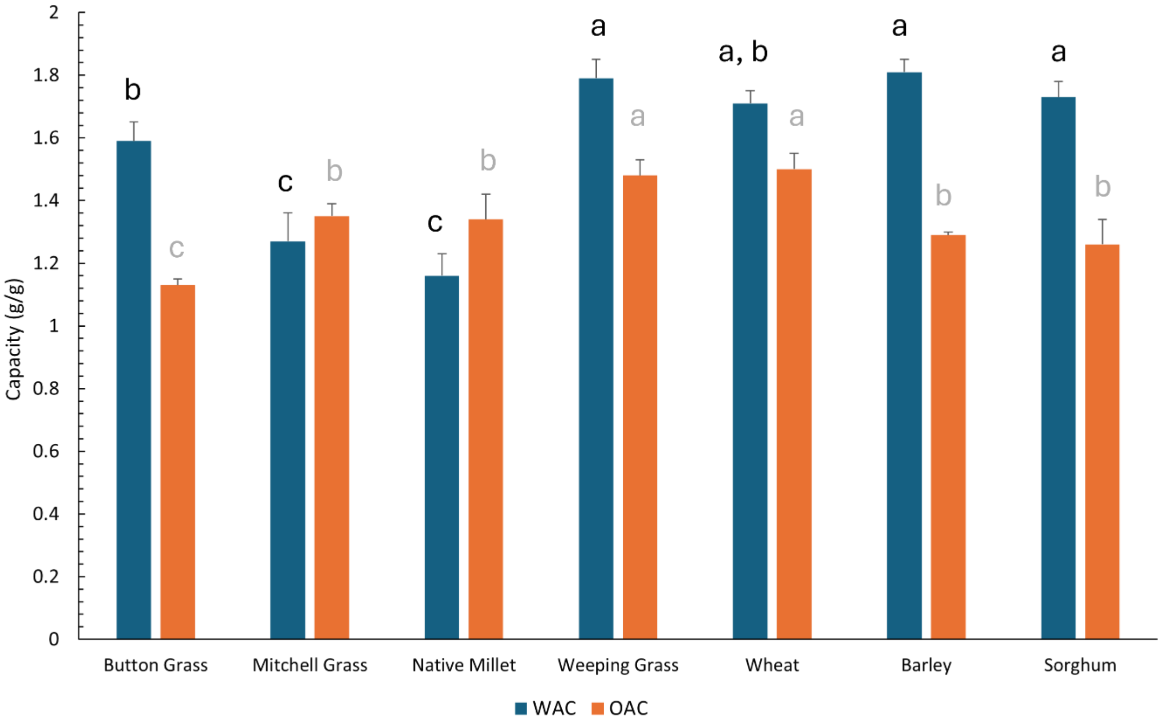

Figure 3.2. Water absorption capacity (g water/g sample) and oil absorption capacity (g oil/g sample) of native grains (Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass) and control grains (wheat, barley, and sorghum). All samples were milled into wholemeal flour from the same batch. Values represent the means of duplicate measurements, with error bars indicating standard deviation (SD). Means with different letters are significantly different ($p < 0.05$), as determined by Tukey's multiple comparisons test following one-way ANOVA.

3.3.7 Bulk Density

Among the native grains, Mitchell Grass had the highest bulk density, followed by Button Grass (Figure 3.3). The lower bulk density in Native Millet and Weeping Grass may result from their coarser particle size, high fibre content, and the presence of substantial bran in Native Millet and β-glucan in Weeping Grass (Adebo & Kesa, 2023; Friday *et al.*, 2023; Rani *et al.*, 2021).

Among the domesticated grains, sorghum exhibited the highest bulk density, marginally surpassing that of wheat. The relatively low bulk density of barley flour may result from its high content of soluble dietary fibre, such as β-glucan, which enhances water absorption and solubility and contributes to a looser flour structure (Rani *et al.*, 2021).

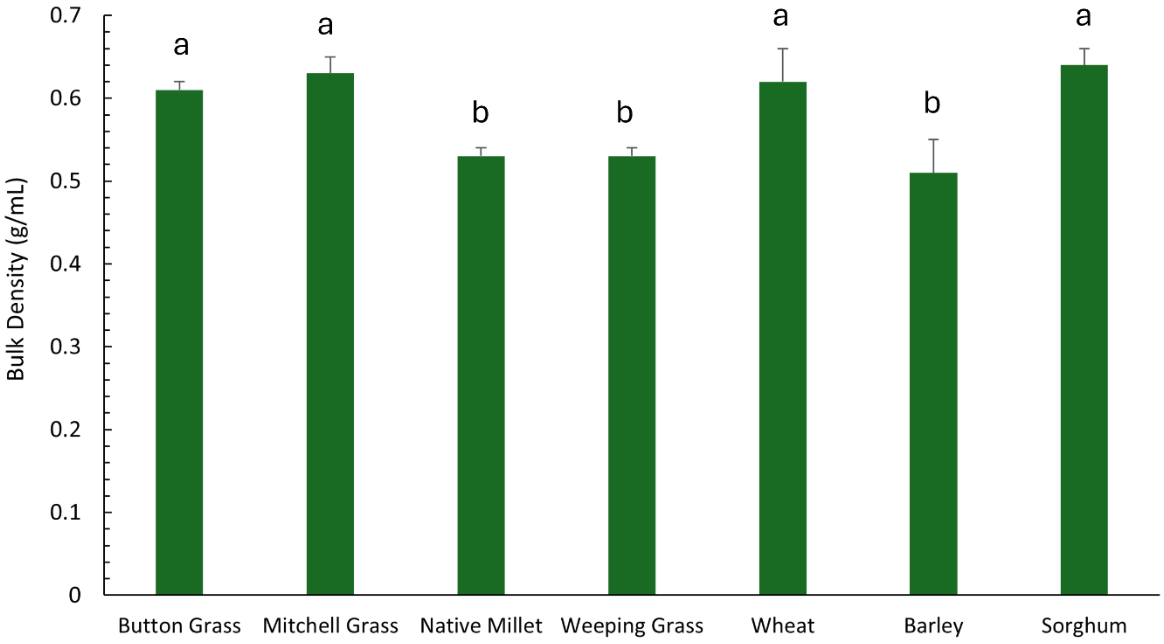

Figure 3.3. Bulk density (g/mL) of wholemeal flours from the native grains (Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass) compared with their domesticated counterparts (wheat, barley, and sorghum). Values represent the mean value of duplicate measurements. Error bars indicate SD. Means with different letters are significantly different ($p < 0.05$), as determined by Tukey's multiple comparisons test following one-way ANOVA.

3.3.8 Foaming Capacity

The foaming capacity of both native and domesticated grain flours was evaluated at pH levels 4, 6, 8, and 10. Among all samples, only wheat flour produced measurable foam across all pH levels, with capacities of 60% at pH 4, 40% at pH 6 and 8, and 56% at pH 10. At pH 10, Mitchell Grass, Weeping Grass, and barley exhibited 30%, 50%, and 20% foaming capacities, respectively. This enhanced foaming capacity at alkaline pH may be attributed to partial deamidation of glutelin and prolamin proteins under these conditions. Alkaline environments are known to promote deamidation, which enhances protein solubility and surface activity—key factors in foam formation and stability (Teklehaimanot & Emmambux, 2019; Zhao, 2011; Zhao *et al.*, 2020).

The unique functional role of the wheat grain storage proteins is their ability to form a viscoelastic network upon hydration, which supports dough structure and stabilises foams by trapping air at the gas–liquid interface (Asrarkulova & Bulushova, 2018; Thewissen *et al.*, 2011). While the soluble albumins and globulins in wheat may support the initial formation of foam, it is the gluten proteins—gliadin and glutenin—that contribute the strength and elasticity required to stabilise and maintain the foam structure. The lack of foaming in the other flours (except for Curly Mitchell Grass, Weeping Grass, and barley under alkaline conditions) may reflect both the absence of gluten-forming proteins and the presence of proteins with limited surface activity or solubility at the tested pH levels.

3.4 Conclusion

Proximate analysis of Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass revealed distinct nutritional profiles. These compositional differences are likely to influence their functional performance in food systems, especially in comparison with domesticated cereals such as wheat, barley, and sorghum.

Wheat is widely recognised as the benchmark for breadmaking and flour-based products, largely due to its balanced protein content, low lipid levels, and high carbohydrate content. Wheat's gluten-forming proteins, gliadin and glutenin, which provide elasticity, extensibility, and gas retention, are essential for the production of leavened bread, pasta, and other baked goods.

All four native grain species were confirmed to be naturally gluten-free (Chapter 5), which restricts their capacity to form viscoelastic dough structures, making them unsuitable for leavened bread production without the incorporation of binders or structure-enhancing agents such as gums, hydrocolloids, or egg proteins. However, their distinct nutrient compositions may contribute to different functional roles in food applications.

The high protein content in Curly Mitchell Grass, for instance, may improve emulsification and nutritional value, and have potential in protein-enriched bakery, snack, and beverage products.

The high lipid content of Native Millet may enhance mouthfeel and richness; however, it also increases the risk of lipid oxidation, which could reduce shelf life. This lipid profile could be beneficial when used in nutrient-dense bars, porridges, or other high-energy formulations.

The high carbohydrate content in Weeping Grass and Button Grass may enhance hydration and gelatinisation behaviour, which supports the use of these grains in cereal-based products such as flakes, extruded snacks, or flatbreads. However, their lack of gluten remains a barrier to use in elastic doughs unless they are combined with wheat or fortified with structural agents.

While native grains may not fully substitute for wheat in many applications, they demonstrate complementary functional properties suited to gluten-free, protein-rich, and energy-dense product development. A clear understanding of their proximate composition allows for more accurate prediction and engineering of processing behaviour, including water absorption, dough consistency, lipid–starch interactions, and nutritional stability.

Certain functional properties of the native grains were consistent with their physicochemical profiles. For example, the high water and oil absorption capacities of Weeping Grass may relate to its elevated β-glucan content, as shown in Chapter 4. The higher dietary fibre and bran content in Native Millet and Mitchell Grass may also have resulted in higher oil absorption and lower bulk density, due to the porous, hydrophilic nature of these components. However, while foam developed across all the tested pH levels only in wheat, two native grains produced foam at pH 10, which suggests that although gluten plays a key role in structure formation, certain native grain proteins (e.g., 12S seed storage globulin 1 in Weeping Grass; Chapter 6) may also support foam formation under alkaline conditions.

Chapter 4

Carbohydrate profiles of Australian native grains

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Applied Food Research

Farkhondeh Abedi, Claudia Keitel, Ali Khoddami, Angela L. Pattison and Thomas H. Roberts

4.1 Introduction

Carbohydrates are normally the primary energy source in human diets and shape the nutritional and functional properties of cereal grains. The carbohydrate composition of grains, including the content of starch, non-starch polysaccharides, and dietary fibre, determines their suitability for various health-focused and industrial food applications. A detailed analysis of carbohydrate profiles can provide insights into the potential of grains for diverse purposes.

Cereal grains are a major source of carbohydrates, which typically constitute between 66% and 76% of their overall composition (Tacer-Caba *et al.*, 2015). The main source of carbohydrates in grains is starch, which exists in a granular form in the endosperm. Starch is primarily composed of amylose (commonly around 15–30%) and amylopectin (Bertoft, 2017). However, mutant genotypes often show variations in the amylose-to-amylopectin ratio. "Waxy" cultivars are characterised by extremely high amylopectin content, often reaching nearly 100%, while high-amylose or "amylostarch" cultivars can contain amylose levels of up to 70% (Varghese *et al.*, 2022). In addition, commercially available high-amylose starches, particularly from maize, can reach amylose levels exceeding 85% (Bird *et al.*, 2007). Amylose is essentially a linear polymer linked by α-1,4 bonds, whereas amylopectin is a highly branched α-1,4 glucan with the branches linked via α-1,6 bonds (Sasaki *et al.*, 2000; Varghese *et al.*, 2022).

Amylose is primarily located in the amorphous regions of starch granules, where it interacts with water and contributes to the disruption of crystalline structures. It also forms complexes with lipids, which influence starch functionality by reducing swelling and enhancing firmness during gel formation (Ee *et al.*, 2020; Sissons, 2016). Amylopectin contributes to granule swelling and viscosity by facilitating water absorption and disrupting crystalline regions more readily during heating. This leads to a lower gelatinisation temperature and higher viscosity compared to amylose-rich starches, which form firmer gels and retrograde more quickly after cooling (Chen *et al.*, 1998; Ee *et al.*, 2020; Zhu, 2018).

A lower glycemic index is commonly observed in high amylose diets. Clinical studies have shown that high-amylose starch intake results in reduced postprandial blood glucose and insulin responses (Li *et al.*, 2024). This response is attributed to the slower digestion rate and lower enzymatic accessibility of amylose compared to amylopectin. Additionally, high-amylose foods may enhance satiety and improve glycaemic control. As a result, individuals with insulin resistance or type 2 diabetes may particularly benefit from these properties (Van Amelsvoort & Weststrate, 1992).

Dietary fibre (DF) refers to plant tissue components that resist enzymatic digestion in the human small intestine (Dhingra *et al.*, 2012). In 2009, the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) established a legal definition of DF as carbohydrate polymers with ten or more monomeric units that are not hydrolysed by human small intestinal enzymes. DF includes three categories: (1) naturally occurring edible carbohydrate polymers, (2) polymers extracted from food by physical, enzymatic, or chemical methods with proven health benefits, and (3) synthetic carbohydrate polymers with demonstrated physiological benefits (Gidley & Yakubov, 2019; Perry & Ying, 2016). It comprises non-starch polysaccharides, resistant oligosaccharides, lignin, RS, dextrin, and synthesised compounds such as polydextrose (Tungland & Meyer, 2002).

DFs are also classified by properties such as source, solubility, and physiological effects. Soluble DF dissolves in water, is easily fermented by gut microflora, and slows digestion, which leads to delayed gastric emptying and slower glucose absorption. It includes pectin, gums, beta-glucan, mucilage, and some non-cellulosic polysaccharides. Insoluble DF does not dissolve in water or form gels and undergoes minimal fermentation. It increases fecal bulk, aids bile acid excretion, and reduces intestinal transit time. Insoluble DF includes cellulose, lignin, and other non-cellulosic polysaccharides (Mudgil, 2017; Williams *et al.*, 2019).

DF plays a crucial role in reducing the risk of cardiovascular disease, improving colonic health and gut motility, and lowering the risk of colorectal cancer. It also correlates with reduced mortality. The gut microbiota mediates many benefits of DF, including appetite regulation, metabolic health, and control of inflammation. Optimising DF intake is a vital public health strategy to improve metabolic and overall health, with significant potential for long-term population benefits (Barber *et al.*, 2020).

An analysis of the effects of DF on dough and bread quality showed that while DF enhanced the nutritional value of bread, it also altered dough properties by increasing water absorption, mixing tolerance, and tenacity while decreasing extensibility. DF extended shelf life but changed sensory properties, and higher levels needed additives to preserve dough rheology (Gómez *et al.*, 2003).

Beta-glucan is a soluble DF commonly found in oat and barley bran. Beta-glucan is predominantly located in the aleurone and sub-aleurone cell walls. Environmental factors affect its levels, which are also controlled by specific enzymes during germination. Barley typically has the highest beta-glucan content among cereals, with between 2 and 20 g/100 g dry weight, of which approximately 65% is water-soluble, followed by oat with a beta-glucan content of 3–8 g/100 g dry weight, of which 82% is water-soluble. Other cereals, such as sorghum, rye, maize, and wheat, contain a much lower beta-glucan content (El Khoury *et al.*, 2012).

Beta-glucans are increasingly recognised for their beneficial effects on insulin resistance, dyslipidemia, hypertension, obesity, and blood glucose regulation, for their ability to lower serum cholesterol levels in diabetic and hypercholesterolemic patients. These benefits stem from beta-glucan's fermentability and its ability to form viscous gut solutions, making it a widely explored ingredient for boosting dietary fibre and health-promoting qualities in foods (Schloermann & Glei, 2017).

Beta-glucan's viscosity, water retention, oil binding, and emulsion stability contribute to its significance in the food industry, where it serves as a thickener and gelling agent in low-fat ice cream, cheese, and sausages, which enhances texture and reduces calorie content. The ability of beta-glucan to form pseudoplastic and thixotropic solutions is critical for creating stable suspensions, sauces, and beverages. Beta-glucan in bakery products like high-fibre bread and pasta enhances viscoelastic properties and sensory qualities, as well as DF content. Additionally, beta-glucan can act as a fat replacer and a carrier for active compounds in functional foods (Guleria *et al.*, 2015).

Resistant starch is a type of starch that resists digestion in the small intestine. This can be attributed to physical inaccessibility, retrogradation, or chemical modification of the starch. RS is recognised as a functional DF by the American Association of Cereal Chemists and by the Food and Nutrition Board of the Institute of Medicine. The numerous health benefits of RS include a reduced risk of colon cancer, haemorrhoids, diverticulosis, and constipation, as well as an increase in faecal bulking. Additionally, it helps control blood glucose and cholesterol levels, acts as a prebiotic, and promotes gut health and better digestive function (Fuentes-Zaragoza *et al.*, 2010; Sharma *et al.*, 2008).

Resistant starch is suitable for a wide range of food products. Its low water-holding capacity and small particle size can enhance firmness and reduce stickiness in pasta by limiting excessive water uptake and maintaining structural integrity during cooking. In baked goods, RS contributes to a finer crumb structure and improved bite due to its neutral flavour, minimal impact on dough rheology, and ability to reduce retrogradation. These functional properties make RS a valuable component in food formulations seeking improved texture and extended shelf life. These functional properties make RS an appealing ingredient for health-oriented food products that keep desirable taste and texture (Nissar *et al.*, 2017; Sharma *et al.*, 2008).

This chapter presents the results of experiments to determine the carbohydrate profiles of four selected Australian native grains and control grains (wheat, barley, and sorghum). The analyses include total starch, total dietary fibre, amylose content, RS, and beta-glucan. The findings contribute to our understanding of the suitability of these grains for health-focused and gluten-free diets and assess their potential for industrial food production.

4.2 Materials and Methods

4.2.1 Plant Materials

The plant materials used in this study were the same as those described in Section 2.2.1 of Chapter 2. Control grains were wheat (*Triticum aestivum* cv. Kord), barley (*Hordeum vulgare* cv. Spartacus), and sorghum (*Sorghum bicolor* cv. Buster), as described in Section 2.2.1. Control grains were analysed as commercial grain lots; detailed agronomic conditions (site, season, inputs) were not available.

4.2.2 Flour Preparation

Whole-grain samples were initially ground for 2 min using a Breville Coffee 'n' Spice Grinder (Model CG2, Issue 2/98; Breville, Australia). However, due to suboptimal results, the coffee grinder was replaced with a Laboratory Mill 3100 (Perten, Australia) fitted with a 0.5-mm screen to achieve a finer and more consistent particle size. The flour samples used in this study were prepared using the same method described in Section 3.2.2 of Chapter 3.

4.2.3 Total Starch Analysis

For total starch content determination, a Total Starch Assay Kit (AA/AMG) from Megazyme (Wicklow, Ireland) was employed. The method involves hydrolytic digestion with α-amylase (AA) and amyloglucosidase (AMG), converting starch to maltodextrins and D-glucose, respectively. This is followed by the use of glucose oxidase-peroxidase (GOPOD) reagent and the measurement of absorbance at 510 nm to quantify the D-glucose (Mccleary *et al.*, 1997).

To apply this method, 30–40 mg of each flour sample was weighed and wetted with 400 µl 80% ethanol, followed by the addition of 2 mL dimethylsulfoxide (DMSO). The mixture was vortexed and heated in a water bath at 100°C for 8 min. Then, samples were heated in a water bath at 100°C for 30 min after adding 100 µl AA, pre-mixed with 3 mL MOPS buffer, and vortexing. After heating, 4 mL of sodium acetate buffer and 100 µl AMG were then added, respectively, and the samples were incubated at 50°C for 30 min. Following incubation, around 1 mL of each sample was transferred to a 1.5 mL centrifuge tube and centrifuged at 2000 × *g* for 10 min (5810/5810 R, Eppendorf, Germany). Then, 3 mL GOPOD reagent was added to 100 µl of each sample in a 25 mL centrifuge tube, followed by incubating in a 50°C water bath in the dark for 20 min. The absorbance was measured at 510 nm against a blank using a spectrophotometer (A Shimadzu UV 1900, Japan).

4.2.4 Amylose Content Analysis

The percentage of amylose in the total starch content of the samples was determined using an Amylose/Amylopectin Megazyme Assay Kit from Megazyme. The method employed lectin concanavalin A (Con A) to precipitate and remove the amylopectin-Con A complex. Subsequently, the remaining amylose molecules were hydrolysed to D-glucose, by using AA/AMG, after which D-glucose was measured by a spectrophotometer at 510 nm with GOPOD reagent (Gibson *et al.*, 1995; Yun & Matheson, 1990).

Following this method, 20–25 mg of the sample was weighed into a 10 mL glass tube, followed by the addition of 1 mL of DMSO. The tubes were vortexed and heated in a boiling water bath for 15 min. After cooling at RT for 5 min, 6 mL of 95% (v/v) ethanol was added, and the mixture was centrifuged at 2000 × *g* for 5 min, after which the supernatant was discarded. The tubes were then boiled again for 15 min, and 4 mL of Con A solvent was immediately added, followed by vortexing. Subsequently, 0.5 mL of Con A solution was added to 1 mL of the sample solution. The sample solutions were centrifuged at 14,000 × *g* for 10 min at RT.

Following the procedure above, 3 mL of 100 mM sodium acetate buffer was added to 1 mL of the supernatant, and the tubes were boiled for 5 min. Then, 0.1 mL of AA/AMG was added, and the tubes were incubated at 40°C for 30 min. The tubes were then centrifuged at 2000 × *g* for 5 min. Then, 1 mL of the supernatant was transferred to a glass tube, and 4 mL of GOPOD reagent was added. The tubes were incubated at 40°C for 20 min, and absorbance was measured at 510 nm using spectrophotometer (A Shimadzu UV 1900, Japan).

4.2.5 Total Dietary Fibre Content Determination

The dietary fibre content of the whole-grain flours was determined using the enzymatic/gravimetric method outlined by Prosky *et al.* (1988), employing a Total Dietary Fiber Megazyme Assay Kit from Megazyme. The tests were run in duplicate for each sample. AA was used to induce gelatinisation, hydrolysis, and depolymerisation of the starch. Protease and AMG were then added to the sample. Soluble fibre was precipitated, and depolymerised proteins and glucose derived from the starch were eliminated. The residue was filtered, dried, and weighed. One of the duplicate samples was subjected to protein analysis, and the other to ash determination. The total dietary fibre was calculated by subtracting the combined weight of the protein and ash from the weight of the filtered and dried residue.

Briefly, 50 mL of sodium phosphate buffer was added to 1 g of each flour sample, followed by 50 µL of AA. The sample solution was then heated in a water bath at 98°C for 15–30 min. The pH was adjusted to 7.5 before adding 100 µL of protease, after which the solution was heated again at 60°C for 30 min. The pH was then reduced to 4.5, 200 µL of AMG was added, and the solution was heated at 60°C for another 30 min. Following this, 200 mL of 95% ethanol was added to the sample solution, and the bottles were left in the dark overnight. The solution was then filtered through a pre-weighed Celite crucible under vacuum and then dried in an oven at 105°C overnight. The dried samples were placed in a muffle furnace (LOVELL-S50, Labec, Australia) at 550°C for 5 h, with a small portion set aside for protein detection. The total dietary fibre content was calculated by the difference between the weight of the dried sample before ashing and the weight of the ash and protein.

4.2.6 Resistant Starch Analysis

The RS content of the flours was determined using a Resistant Starch Rapid Kit from Megazyme. This method is an update of the procedure developed by McCleary *et al.* (AOAC Method 2002.02; AACC Method 32-40.01), employing incubation conditions similar to those used in AOAC Method 2017.16 for dietary fibre (McCleary *et al.*, 2002). Pancreatic AA and AMG were employed to solubilise and hydrolyse non-RS into D-glucose. RS was recovered as a pellet by centrifugation. The pellet was dissolved in 1.7 mol L^{-1} NaOH and neutralised with acetate buffer (pH 3.8). AMG was utilised to hydrolyse the starch to D-glucose, followed by the use of GOPOD reagent and measuring the absorbance at 510 nm to determine the D-glucose (McCleary *et al.*, 2002).

To begin the RS analysis, 3.5 mL of sodium maleate buffer was added to 100 mg of the flour sample, and the solution was incubated in a 37°C water bath for at least 5 min to reach temperature equilibrium. Subsequently, 0.5 mL of PAA (porcine pancreatic α-amylase)/AMG in sodium maleate buffer was added to the tubes, followed by incubation in a 37°C water bath with continuous horizontal shaking for 4 h. Then, 4 mL of 95% (v/v) ethanol was added to each tube, and the samples were centrifuged at 4000 rpm for 10 min. The resulting pellet was resuspended in 8 mL of 50% (v/v) ethanol, vortexed vigorously, and centrifuged again at 4,000 rpm for 10 min. The pellet was then resuspended (in 2 mL of 1.7 M NaOH) and stirred in ice for 20 min. Subsequently, 8 mL of 1 M sodium acetate buffer and 0.1 mL of AMG (3,300 U/mL) were added. The tubes were incubated at 50°C for 30 min and then centrifuged at 4,000 rpm for 10 min. To determine glucose concentration, 3 mL of GOPOD reagent was mixed with 0.1 mL aliquots of the supernatant, standards, and a reagent blank, and the tubes were incubated at 50°C for 20 min. Finally, the absorbance at 510 nm was recorded against the blank using spectrophotometer (A Shimadzu UV 1900, Japan).

4.2.7 Beta-glucan Determination

A Mixed Linkage β-glucan Kit from Megazyme was used for beta-glucan (β-D-glucan) determination. Samples were incubated with lichenase enzyme, followed by hydrolysis with β-D-glucosidase. Lichenase specifically breaks down β-D-glucan into oligosaccharides, which are hydrolysed into glucose by β-glucosidase. The resulting glucose concentration was determined by measuring the absorbance at 510 nm using the GOPOD reagent (McCleary & Codd, 1991).

A 100-mg flour sample was wet with 0.2 mL of 50% (v/v) ethanol, mixed vigorously, and then combined with 4 mL of 20 mM sodium phosphate buffer. The sample solution was incubated in boiling water for 2 min, followed by incubation at 50°C for 5 min. Next, 0.2 mL of lichenase enzyme was added, and the tubes were incubated at 50°C for 1 h. After adding 5 mL of 200 mM sodium acetate buffer, the tubes were centrifuged at 1000 × *g* for 10 min. A 0.1-mL aliquot of the supernatant was then mixed with 0.1 mL of β-glucosidase enzyme and incubated at 50°C for 10 min. Subsequently, 3 mL of GOPOD reagent was added to all tubes (sample, glucose standard, and blank), and the tubes were incubated at 50°C for 20 min. Absorbance was measured at 510 nm against a blank spectrophotometer (A Shimadzu UV 1900, Japan).

4.2.8 Statistical Analysis

For sample characterisation, measurements were performed in triplicate for each flour sample from each of the species. This denotes that three separate measurements were taken for each individual flour sample, and the process was repeated for all species in the study. All data represent the mean ± standard deviation. Data were analysed using GraphPad Prism v.10.2.1 (GraphPad Software, San Diego, CA, USA). A standard one-way analysis of variance (ANOVA) was conducted to assess differences among the tested groups for all measured parameters. Tukey's *post hoc* multiple comparisons test was performed to identify significant differences between groups. Statistical significance was determined at $p < 0.05$.

4.3 Results and Discussion

4.3.1 Flour Preparation and Method Optimisation

Initial total starch analysis of samples ground with a coffee grinder produced unsatisfactory results in two separate experiments (Table 4.1). To investigate the cause, microscopy images of the flour produced by the coffee grinder and the Laboratory Mill 3100 were captured and compared. The mill flour exhibited a finer and more uniform particle size, with an average of approximately 0.5 mm (Figure 4.1). Following this observation, the milling method was adjusted, the laboratory mill with a 0.5-mm screen size was used, and total starch analysis was repeated. The results for the coffee grinder-prepared flour are presented in Table 4.1.

Table 4.1. Total starch content (g/100 g, wet basis) of whole-grain samples, ground with a coffee grinder for 2 min.

Species	**Total starch**	
	Experiment 1	**Experiment 2**
Button Grass	38.1 ± 1.0	39.3 ± 0.8
Curly Mitchell Grass	44.0 ± 0.3	45.3 ± 1.2
Native Millet	48.7 ± 0.4	39.8 ± 0.5
Weeping Grass	45.4 ± 0.3	47.5 ± 1.4
Wheat	67.4 ± 0.5	54.8 ± 1.1
Barley	55.8 ± 0.2	64.9 ± 0.7
Sorghum	64.3 ± 1.2	67.5 ± 0.6

Data are presented as the mean ± standard deviation of three independent determinations, using wholemeal flour from the same batch of grains (n=3)

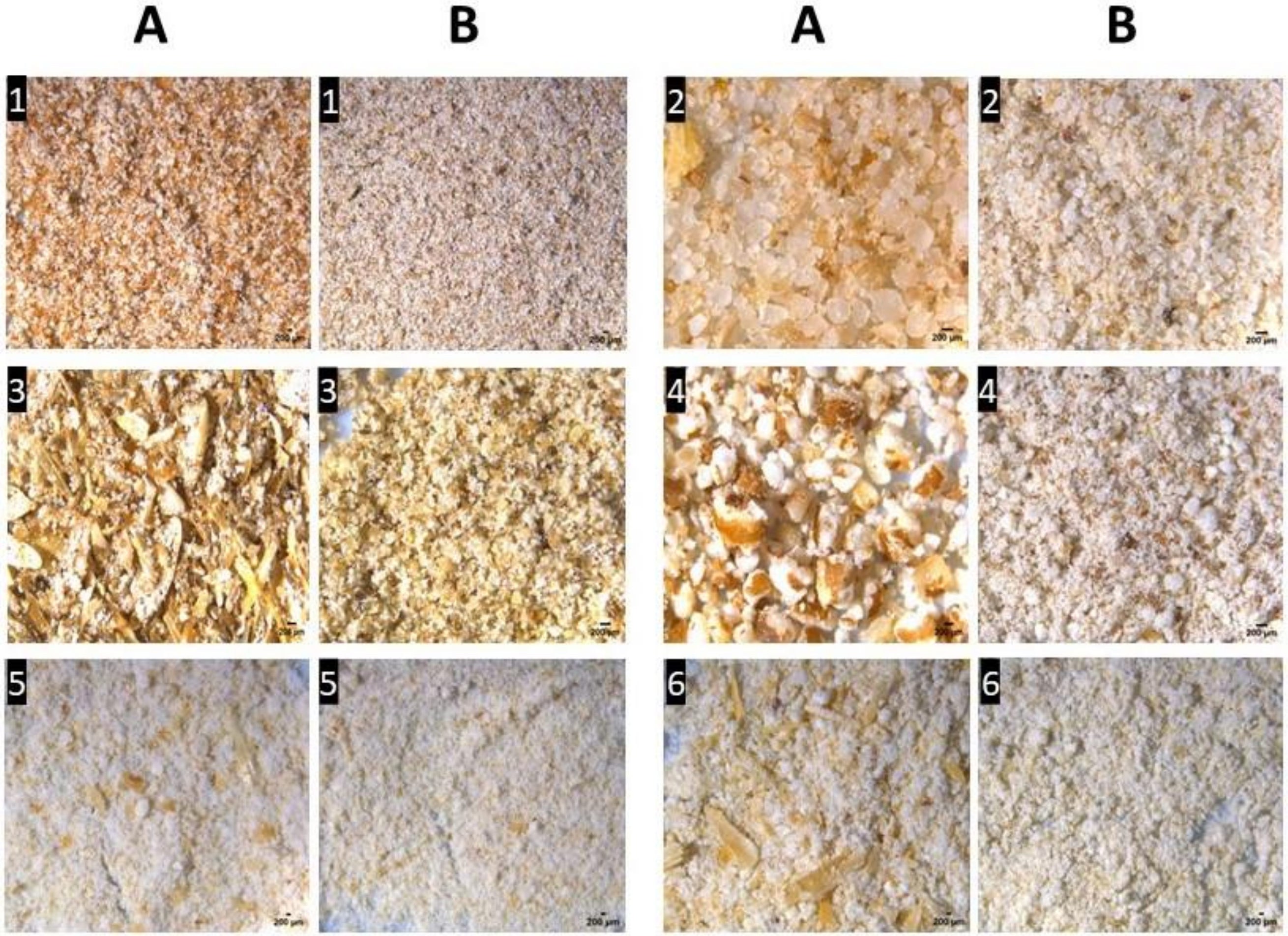

Figure 4.1. Microscopic comparison of flour particle size and uniformity from whole-grain samples of 1. Button Grass, 2. Curly Mitchell Grass, 3. Native Millet, 4. Weeping Grass, 5. wheat, and 6. barley, processed using a coffee grinder (A) and a Laboratory Mill 3100 (B). Scale bar = 200 µm.

4.3.2 Total Starch Content

The total starch content among the studied grains was substantially lower on average than their domesticated counterparts (Table 4.2). The results were comparable to the pattern reported by Brand-Miller *et al*. (1998), in which native grains contained 45.0 ± 2.0 g/100 g carbohydrate (combined starch and sugar). Our native grain total starch values, which ranged from 45.8 ± 0.4 g/100 g wb (53.7 ± 0.4 g/100 g db) for Native Millet to 53.5 ± 0.3 g/100 g wb (61.8 ± 0.3 g/100 g db) for Weeping Grass, were considerably lower than that reported for Kangaroo Grass (62.1 ± 0.8 g/100 g wb; Cowley *et al*., 2023).

This lower starch content likely reflects relatively higher protein, lipid and fibre contents, as commonly reported for small-seeded grains such as millets, and is associated with favourable nutritional properties, including lower glycaemic responses (Saleh *et al.*, 2013). However, these same compositional features can limit performance in conventional starch-rich products (e.g., bread and noodles) unless blended with wheat or supported by processing adjustments, as shown in millet-wheat systems (Shukla & Srivastava, 2014).

Table 4.2. Total starch, amylose, total dietary fibre, resistant starch, and beta-glucan content of the native and control grains. All values are expressed as g/100 g db of the grains except for amylose content, which is expressed as g/100 g of total starch.

Species	Total starch	Amylose	Total dietary fibre	Resistant starch	Beta-glucan
Button Grass	54.9 ± 1.0 ^{d}	25.7 ± 0.6 ^{c}	21.9 ± 0.7 ^{a}	0.7 ± 0.0 ^{e}	0.2 ± 0.0 ^{d}
Curly Mitchell Grass	57.7 ± 0.3 ^{c}	41.2 ± 1.3 ^{a}	14.6 ± 0.3 $^{c, d}$	2.8 ± 0.2 ^{d}	0.3 ± 0.0 ^{d}
Native Millet	53.7 ± 0.4 ^{d}	41.0 ± 0.7 ^{a}	14.3 ± 0.3 ^{d}	3.0 ± 0.0 ^{d}	0.1 ± 0.0 ^{d}
Weeping Grass	61.8 ± 0.3 ^{b}	31.8 ± 0.9 ^{b}	16.8 ± 0.4 ^{b}	5.9 ± 0.0 ^{c}	8.6 ± 0.3 ^{a}
Wheat	78.3 ± 0.5 ^{a}	20.9 ± 0.7 ^{d}	11.4 ± 0.2 ^{e}	3.1 ± 0.2 ^{d}	0.9 ± 0.1 ^{c}
Barley	62.8 ± 0.2 ^{b}	31.1 ± 0.1 ^{b}	15.5 ± 0.3 ^{c}	8.3 ± 0.6 ^{a}	4.2 ± 0.2 ^{b}
Sorghum	77.1 ± 1.2 ^{a}	21.3 ± 0.5 ^{d}	9.5 ± 0.2 ^{f}	6.9 ± 0.4 ^{b}	1.1 ± 0.0 ^{c}

Data are presented as mean ± standard deviation of three independent determinations, using wholemeal flour from the same batch of grains (n=3). Means within each column followed by different letters are significantly different ($p < 0.05$), as determined by Tukey's multiple comparisons test following one-way ANOVA.

4.3.3 Amylose Content

The analysis of amylose content in the studied grains revealed notable variations in their starch composition. Curly Mitchell Grass and Native Millet, with amylose contents of 41.2 ± 1.3 and 41.0 ± 0.7 g/100 g of the total starch, respectively (Table 4.2), had the highest amylose content among the native and control grains ($p < 0.05$), but not as high as, for example, high-amylose maize (Obadi *et al.*, 2023). The high amylose content observed in these species might contribute to a relatively low glycaemic index, as amylose is closely linked to slowly digestible starch in cooked grains (Cowley *et al.*, 2023).

Weeping Grass, with an amylose content of 31.8 ± 0.9 g/100 g of the total starch, aligned more closely with the control species, particularly barley (31.1 ± 0.1 g/100 g). Button Grass had the lowest amylose content among the native grains (25.7 ± 0.6 g/100 g), but somewhat higher than that observed in wheat (20.9 ± 0.7 g/100 g) and sorghum (21.3 ± 0.5 g/100 g) (Table 4.2).

The amylose content of the wheat sample measured here (20.9%) is lower than the average reported for bread wheat starch (~25%) (Li *et al.*, 2023), but still within the expected range. For example, Slade *et al.* (2012) reported ~23% amylose in wild-type wheat, while Hallstrom *et al.* (2011) noted values as low as ~20% in wheat starch blends (Hallström *et al.*, 2011; Slade *et al.*, 2012).

How the ratio of amylose to amylopectin in the native grains will influence bread-making and the production of other grain-based foods will need to be investigated in the future. Amylose inhibits water absorption, and participates in the plasticisation of starch granules by interacting with water, disrupting crystalline structures, and forming complexes with lipids, which makes the starch softer and more flexible (Ee *et al.*, 2020; Sissons, 2016). Amylopectin influences the crystallinity of starch, affecting properties like water insolubility, swelling, and viscosity, depending on its internal molecular structure (Ee *et al.*, 2020; Zhu, 2018).

4.3.4 Total dietary fibre

The total dietary fibre content of Weeping Grass (16.8 ± 0.4 g /100 g db) was similar to that of barley (Table 4.2). Curly Mitchell Grass exhibited a total dietary fibre content of 13.1 ± 0.2 g/100 g wet basis (wb), equivalent to 14.6 ± 0.3 g/100 g db, which is comparable to the results reported by Foster *et al.* (2010), who found a total dietary fibre content of 15.0 g/100 g wb for Curly Mitchell Grass. Button Grass demonstrated a notably higher total dietary fibre content (21.9 ± 0.7 g/100 g db) compared to barley and the other native grains analysed (Table 4.2; $p < 0.05$). While this elevated fibre level adds nutritional value, excessive fibre can negatively affect food quality by reducing dough extensibility, producing denser textures, and potentially lowering consumer acceptability (Elleuch *et al.*, 2011; Gómez *et al.*, 2003).

A higher total dietary fibre content can enhance the potential for breadmaking by increasing water absorption and mixing tolerance while decreasing extensibility. The higher fibre content may also extend the shelf life of bread due to its ability to retain moisture and slow starch retrogradation, which helps delay crumb firming and staling (Bagheri & Seyedein, 2011; Gómez *et al.*, 2003). Moreover, the consumption of grains rich in dietary fibre is linked to reduced risks of non-communicable diseases, including cardiovascular diseases, cancers, gastrointestinal disorders, obesity, and type 2 diabetes (Barber *et al.*, 2020; Buttriss & Stokes, 2008; Gill *et al.*, 2021; Hojsak *et al.*, 2022; Joye, 2020).

As wholemeal flour was analysed, the dietary fibre values reflect contributions from both bran and endosperm fractions. The comparatively high fibre content of Button Grass indicates a larger contribution from outer grain tissues, consistent with reports on small-seeded cereals, where the bran fraction accounts for much of the dietary fibre (Saleh *et al.*, 2013). In contrast, the fibre in Weeping Grass may include a higher proportion of cell-wall polysaccharides such as β-glucans, which are important contributors to dietary fibre in cereals (Saulnier *et al.*, 2007).

4.3.5 Resistant starch (RS)

The native grains in this study consistently displayed lower levels of RS compared to the control grains, particularly wheat (Table 4.2). The low RS content of Button Grass (0.6 ± 0.0 g/100 g wb, equivalent to 0.7 ± 0.0 g/100 g db) was similar to that reported in a previous study for Kangaroo Grass (0.9% w/w wb; Cowley *et al.*, 2023).

RS values are reported as means ± SD of analytical replicates (n = 3). For some grains, including Button Grass and Weeping Grass, the SD was < 0.05 g/100 g and therefore rounded to 0.0 at one decimal place. This reflects reporting precision rather than a complete absence of variation.

Among the native grains, Weeping Grass exhibited the highest level of RS (5.9 ± 0.0 g/100 g db), which indicates its potential for incorporation in baked goods, pasta, and beverages to improve texture and provide health benefits. Breads with an optimum RS content exhibit increased crumb moisture, reduced firmness, and a lower retrogradation rate (Barros *et al.*, 2018). The latter, coupled with the higher crumb moisture and water-retention capability of RS, contributes to an overall reduction in crumb firmness in these breads. Resistant starch is also known to prolong the freshness of bread (Arp *et al.*, 2021; Mohebbi *et al.*, 2018).

Weeping Grass also exhibited potential for utilisation in thickened, opaque health beverages due to the swelling capacity and ability to form gels of RS. In contrast to insoluble fibre, RS provides a smoother mouthfeel and has a milder impact on sensory properties such as flavour (Fuentes-Zaragoza *et al.*, 2010; Sharma *et al.*, 2008; Tabibloghmany & Ehsandoost, 2014).

4.3.6 Beta-glucan

The beta-glucan contents of the native grains in this study were higher than those reported for Kangaroo Grass (0.05 ± 0.0%, w/w) (Cowley *et al.*, 2023). Weeping Grass displayed a higher beta-glucan content (8.6 ± 0.3 g/100 g db) compared to the other native grains or compared across both the native and control grains ($p < 0.001$; Table 4.2). This finding builds on the microscopy-based study in which it was identified that Weeping Grass had thicker endosperm cell walls than the other species investigated (Abedi *et al.*, 2023), as evidenced by blue staining with calcofluor white, indicative of beta-glucan content.

Beta-glucan is an important dietary component that plays a key role in the prevention of cardiovascular diseases, the management of obesity and diabetes, and the regulation of cholesterol levels in the body (Ciecierska *et al.*, 2019; EFSA Panel on Dietetic Products & Allergies, 2010; Eraniappan *et al.*, 2023; Niazi *et al.*, 2023; Yu *et al.*, 2022). Using beta-glucan for its functional properties in foods, including thickening, stabilising, emulsifying, and gelling, offers opportunities to enhance ingredient stability, particularly in the production of beverages. When beta-glucans are added to various products, such as bread, muffins, pasta, noodles, salad dressings, beverages, soups, and reduced-fat dairy and meat items, their attributes become apparent. These include breadmaking performance, water binding, emulsion stabilising capacity, thickening ability, texture, and appearance, which are linked to the concentration, molecular weight, and structure of this polysaccharide (Din *et al.*, 2018; Kaur *et al.*, 2019; Lazaridou & Biliaderis, 2007). Beta-glucan is found to extend the shelf life of bread while reducing its tendency to become stale (Mohebbi *et al.*, 2018).

4.4 Conclusion

Australian native grains demonstrated distinct nutritional and functional characteristics in total starch, amylose, dietary fibre, RS, and beta-glucan content compared to domesticated grains. These characteristics indicate their potential for both improved nutritional value and functional applications in food production. Weeping Grass, for instance, exhibited the highest beta-glucan content among all studied grains, underscoring its strong potential for a variety of food industry applications, including improving bread texture and shelf life and enhancing the viscosity and stability of health beverages. Similarly, Curly Mitchell Grass and Native Millet displayed high amylose content, indicating their suitability for developing low-glycaemic index foods.

The native grains studied offer a valuable combination of high amylose content, substantial dietary fibre, and functional polysaccharides such as beta-glucan and RS, which may enhance both the nutritional quality and processing performance of food products. These attributes reflect increasing consumer interest in food ingredients that are health-promoting, sustainable, and functionally beneficial.

The integration of these grains into contemporary food applications presents an opportunity to expand carbohydrate sources apart from conventional cereals. This research helps develop health-oriented products for dietary requirements like gluten-free and low-GI diets and extends the range of nutritional options by exploring underutilised native species. Their unique carbohydrate profiles make them suitable for products aligned with consumer interest in health, wellness, and sustainability.

The structural properties of the carbohydrates in these native grains may also influence their behaviour during processing and storage. Various amylose-to-amylopectin ratios, for example, can impact gelatinisation, retrogradation, and water absorption, crucial for product texture, shelf life, and sensory quality. High amylose content, for instance, may contribute to firmer textures and improved thermal stability, while beta-glucan and RS may boost viscosity and water-binding capacity. Understanding these features is essential for optimising their use in processed food applications.

Moreover, adding these grains to food products could support the creation of functional foods with health benefits beyond basic nutrition. These physiological effects position native grains as promising ingredients in foods aimed at improving metabolic health, including diabetes management, weight control, and digestive well-being. Further study of their carbohydrate content and properties could lead to new ideas for product development in the health and wellness sector.

Chapter 5

Protein analysis of Australian native grains

This chapter is an extended version of part of a revised manuscript submitted to

Applied Food Research

Farkhondeh Abedi, Claudia Keitel, Ali Khoddami, Angela L. Pattison and Thomas H. Roberts

5.1 Introduction

The protein composition of cereal grains partly determines their nutritional quality, functionality, and suitability for food applications. Proteins influence the functional properties of cereal grains in food formulations and affect the texture, elasticity, and water absorption capacity of bakery products (Lasztity, 2017; Shewry & Halford, 2002).

According to the Osborne system, the proteins of cereals can be categorised into four fractions based on their solubility in various solvents: albumins, globulins, prolamins, and glutelins (soluble in water, dilute salt solutions, aqueous alcohol, and dilute acids or bases, respectively). Most albumins and globulins are metabolic proteins like enzymes, enzyme inhibitors and transcription factors and are found in the aleurone layer, bran, and germ. Prolamins and glutelins are concentrated in the starchy endosperm and are broken down to amino acids to feed the growing embryo during germination (Chen *et al.*, 2025; Koehler & Wieser, 2013).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins based on molecular weight. Upon heating, SDS unravels protein structure and binds to the polypeptides, giving them a uniform negative charge, which masks their native charge. Thus, when subjected to an electric field, the proteins migrate through the polyacrylamide gel according to size (larger the slower). This method allows direct comparison of protein size and relative abundance across samples (Laemmli, 1970).

Osborne fractionation and SDS-PAGE aid in understanding grain protein functionality and help determine how processing techniques such as milling, heat treatment, and fermentation modify the protein component of the flour or food. Understanding these protein characteristics will facilitate the development of innovative food applications and improve the commercial viability of the native grains (Shewry & Halford, 2002).

Although the protein profiles of domesticated grains are well documented, the protein composition of underutilised grains, including Australian native species, remains largely uncharacterised (Alvarez-Jubete *et al.*, 2010).

This chapter reports experiments aimed to investigate the protein composition of four Australian native grains by applying gluten screening, Osborne fractionation, and SDS-PAGE analysis. This research provides a foundation for assessing the potential of wholemeal flours from native grains as functional ingredients in gluten-free and nutritionally enhanced food products by comparing their protein profiles with those of domesticated cereals. Analysing protein fractions and molecular weight distributions helps to understand the potential of native wholemeal flour in applications in the bakery, pasta, and alternative protein industries, expands the use of alternative grains in the food industry, supports sustainability, and encourages the adoption of native grains in widely used food systems.

5.2 Materials and Methods

5.2.1 Plant Materials

The plant materials used in this study were the same as those described in Section 2.2.1 of Chapter 2. Barley, one of the reference grains described in Section 2.2.1 of Chapter 2, was used as the control in this study.

5.2.2 Flour Preparation

Flour preparation followed the same procedure described in Section 3.2.2 of Chapter 3.

5.2.3 Gluten Screening

Native and control grains (including brown rice as a gluten-free control) were analysed using a 3M™ Gluten Protein Rapid Kit according to the manufacturer's instructions. This kit employs a lateral flow device (LFD) based on an immunochromatographic test method utilising a polyclonal antibody specifically designed for detecting gliadin in grain proteins of wheat, secalins in rye, and hordeins in barley. The ground sample (0.2 g) was transferred to a microcentrifuge tube. Extraction Buffer (1.8 mL of 3 mol. L^{-1}) was added, followed by vigorous vortexing for 3 min. The extracted sample was centrifuged at 2,350–4,600 *g* for 20–30 s, with the supernatant collected as the extracted sample.

An aliquot (100 µL) of the extracted sample was transferred to the designated sample well on the LFD (the wheat sample was diluted in a 1:3 ratio with the extraction buffer due to its high viscosity). After 10 min, the sample was regarded as positive for gluten if both the test and the control lines were visible on the LFD. If only the control line was visible on the LFD, the sample was considered gluten-free. Additionally, any faint appearance of the test line was interpreted as a negative result, indicating the absence of detectable levels of gluten protein in the sample. The images were recorded employing a Nikon D3400 digital single-lens reflex camera (designed by Nikon Corporation in Japan, manufactured in Thailand).

5.2.4 Protein Fractionation via the Osborne Method

A modified Osborne fractionation procedure was conducted to extract proteins from the native grains and barley (as a control) wholemeal flours, based on Osborne's 1907 method (Singh *et al.*, 2019; Van de Vondel *et al.*, 2020). In each extraction, 1 g of the flour was suspended in 10 mL of Milli-Q water to solubilise the albumins and mixed for 30 min at 100 revolutions per minute (rpm) using an Intelli-mixer RM-2 (John Morris, Australia) in "UU" mode, followed by centrifugation at 2,600 *g* for 20 min using an Eppendorf centrifuge 5810/5810 R. The supernatant was removed as the sample for further analysis.

The pellet was then dissolved in another 10 mL of Milli-Q water, mixed, and the suspension centrifuged as above. These steps were then repeated a third and fourth time. Following this semi-exhaustive extraction of the albumins, the pellet was suspended in 10 mL of 0.4 mol. L^{-1} sodium chloride solution to solubilise the globulins, and the mixing and centrifugation steps were repeated three more times. After the final round of salt extraction, the supernatant was retained, and the pellet was suspended in 10 mL of 70% (v/v) ethanol to solubilise the prolamins, with the same mixing and centrifugation procedure applied. Finally, the procedure was repeated with 10 mL of 0.1 mol. L^{-1} sodium hydroxide added to the pellet to solubilise the glutelins.

In each of the four rounds of extraction for the four types of solvent, the resulting supernatants were then pooled for protein content determination using the bicinchoninic acid assay (BCA assay, Thermo Scientific Pierce^{TM}, Rockford, IL, USA) following the manufacturer's instructions, employing a UV-1600PC spectrophotometer (VWR, USA). Thus, estimates of the absolute and relative abundances of albumins, globulins, prolamins, and glutelins in the wholemeal flour samples were obtained.

5.2.5 Characterisation of the Protein Profiles of Each Fraction

Protein profiles of each Osborne fraction were analysed using SDS-PAGE. Albumin, globulin, prolamin, and glutelin fractions extracted from the native grains and barley were diluted to the lowest protein fraction concentration (the globulin concentration in Button Grass: 0.263 mg/mL). SDS-PAGE was performed using a Bio-Rad Mini-PROTEAN Tetra Vertical Electrophoresis Cell apparatus and 4–20% precast polyacrylamide Mini-PROTEAN TGX gels (1 mm thick; Bio-Rad Laboratories, USA). The gels were run at 200 V (constant voltage) for

35 min until the bromophenol blue marker had reached the bottom of the gels. Replicate gels were stained with either Coomassie Brilliant Blue R-250 or silver nitrate.

For sample preparation, 12 µL of protein extract was mixed with 4 µL of sample buffer (Bio-Rad Tris-glycine-based 4× sample buffer), resulting in a total volume of 16 µL. The mixture was heated at 70°C for 10 min in an Eppendorf tube using an Eppendorf Thermomixer Compact 5350 (Eppendorf, Germany).

Following sample preparation, 12 µL of the extract buffer mixture was loaded into each gel well. Bio-Rad PAGE Ruler Plus pre-stained molecular weight standards were used as molecular weight markers, with lane 1 and lane 15 dedicated to the standards (3 µL per lane).

For whole protein visualisation, seed flour was dissolved in sample buffer at a ratio of 1:10 (w/v), with the addition of 80 µL of 1 M dithiothreitol (DTT) per 1 mL of buffer. The suspension was then centrifuged at 3,500 rpm for 30 min, followed by heating at 70°C for 10 min. After preparation, 12 µL and 6 µL of each sample were loaded onto the gel. The marker was diluted 1:5 in the sample buffer before loading.

For the Coomassie Brilliant Blue staining, the staining and destaining protocol involved two main solutions: the Coomassie Brilliant Blue R-250 dye and the Super Destaining Solution. To prepare 100 mL of Coomassie Brilliant Blue R-250 dye, 100 mg of Coomassie R-250 dye was added to 90 mL of the Super Destaining Solution and mixed thoroughly with a magnetic stirrer until the dye was completely dissolved. The Super Destaining Solution was composed of glacial acetic acid, methanol, and Milli-Q water in a ratio of 1:4:5 (v/v/v).

The gel was immersed in the Brilliant Blue R-250 Staining Solution and then subjected to microwave heating at high power (30–60 s), ensuring that the solution did not boil (Thermo Fisher Scientific, n.d). Subsequently, the stained gel was destained by microwave heating at high power (30–60 s) in the Super Destaining Solution until boiling. The gel was further incubated in the Super Destaining Solution for 1 h to enhance band visualisation.

For the silver nitrate staining, the gels were incubated in a solution of 25% ethanol, 1% HNO_3, and 0.2% $AgNO_3$ for 5–10 min with gentle agitation. The gels were then rinsed with distilled water for 3 min to remove excess stain and impurities and incubated in the development solution (3% Na_2CO_3 and 0.2% HCOH) for 2–5 min at room temperature with gentle agitation. In the last step, the gels were incubated in distilled water for 2 min to halt the staining process.

Images of the gels were recorded using a ChemiDoc MP imaging system. Background subtraction for the gel images was conducted using ImageJ software. Additionally, brightness and contrast adjustments were applied using the “Auto” function in ImageJ to enhance visualisation.

5.2.6 Statistical Analysis

For Osborne fractionation, measurements were performed in triplicate for each flour sample from each species. All results are presented as mean ± standard deviation.

A one-way analysis of variance (ANOVA) was conducted using GraphPad Prism v.10.2.1 (GraphPad Software, San Diego, CA, USA) to determine significant differences among species for each protein fraction. Statistical significance was defined as $p < 0.05$.

SDS-PAGE analysis qualitatively compared the polypeptide profiles of albumin, globulin, prolamin, and glutelin fractions across species. SDS-PAGE results were interpreted based on visual assessment of band patterns.

5.3 Results and Discussion

5.3.1 Gluten Screening

Based on the results, all the studied native grains—Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass—were gluten-free, as indicated by the absence of a test line on the LFD (Figure 5.1). Among the control grains, only wheat and barley tested positive for gluten, which is consistent with their known gluten content.

The observed faintness and incompleteness of the control band on the LFD for the barley sample can be attributed to the viscosity of the sample. The undiluted barley extract impacted the mobility of the buffer within the device, impeding its ability to ascend effectively to the top of the LFD. Conversely, sorghum and brown rice, also part of the control group, tested negative (Figure 5.1), aligning with their established gluten-free status (Ellis *et al.*, 1998). The pink background observed on the LFD for the sorghum samples is caused by a natural red pigment present in the grain extract.

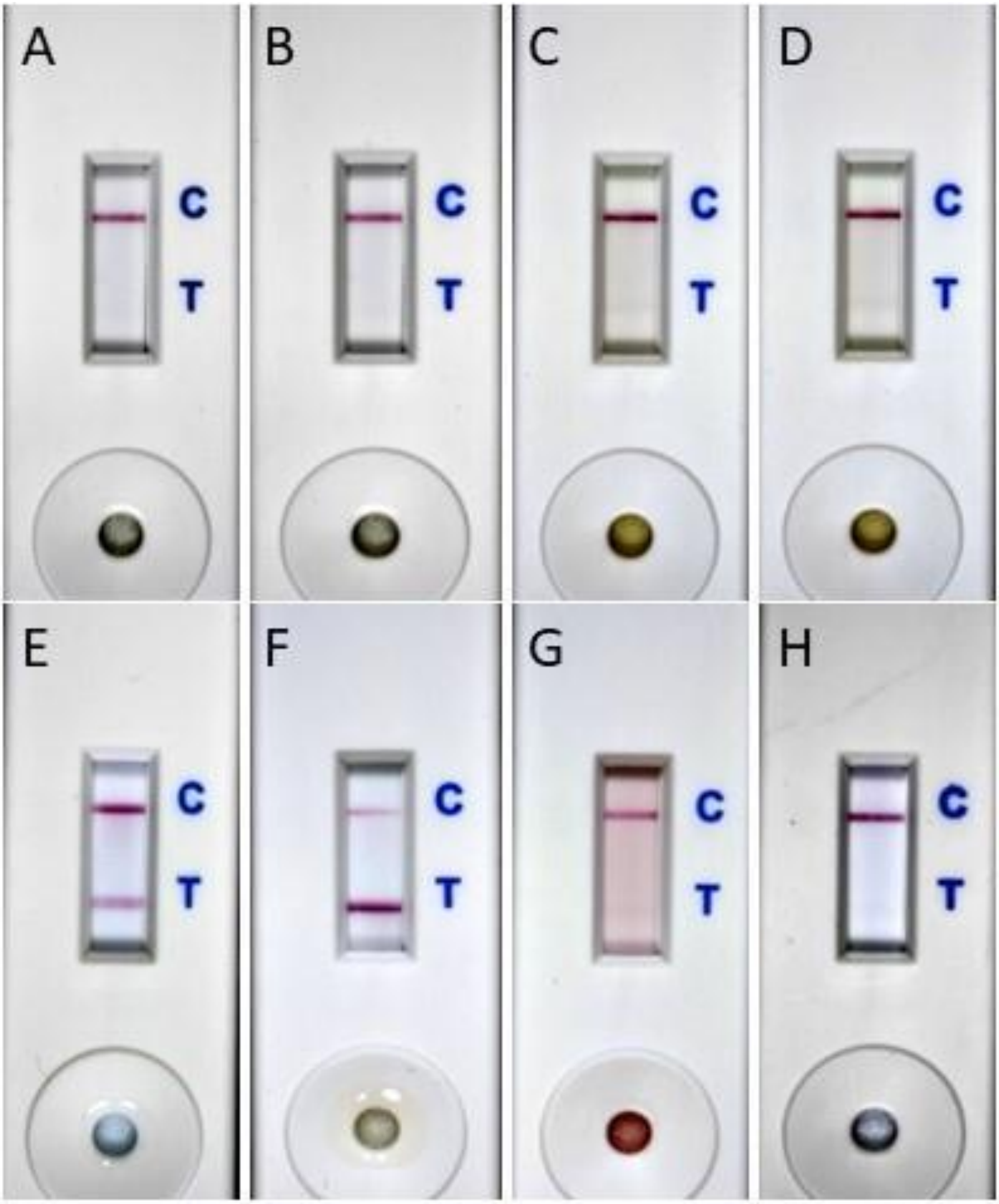

Figure 5.1. The presence or absence of gluten in native and control grains was determined using a lateral flow device. A sample was considered positive for gluten if both the test and control lines were visible on the LFD; the absence of the test line or only a faint line indicated a negative result. The images are presented across eight panels: A (Button Grass), B (Curly Mitchell Grass), C (Native Millet), D (Weeping Grass), E (wheat), F (barley), G (sorghum), and H (brown Rice).

5.3.2 Osborne Protein Fractionation

The results uncovered variation in the proportions of the Osborne protein fractions in the native grain species, with barley as a control (Figure 5.2). Among the studied grains, glutelin content was consistently the dominant fraction, except in Native Millet, for which the albumins were dominant. Weeping Grass had the highest abundance of glutelin (51.8% of the total, including albumins, globulins, prolamins, and glutelins).

The abundance of globulin was the lowest among the protein fractions across the native and control grain species, with Button Grass consistently showing the lowest globulin proportion (2.2% of the total) ($p < 0.05$) compared to the other studied grains. The only exception was Weeping Grass, for which globulins represented 17.4% and the prolamin fraction represented the lowest proportion of total protein (7.1%).

In the microscopy study of the same four Australian native grains (Chapter 2), 2,3,5-triphenyltetrazolium chloride solution was used to highlight the embryo in longitudinally cut grains (orange to red staining) and acid fuchsin to highlight protein in longitudinal sections (red staining) (Abedi *et al.*, 2023). In that research, Curly Mitchell Grass and Native Millet were estimated to have a larger embryo as a proportion of the total grain compared with the other species (including sorghum and wheat), with concentrated protein observed in the aleurone and sub-aleurone layers of the longitudinal sections.

In the present investigation, the albumin fraction, which is known to be mainly distributed in the embryo and aleurone layers of cereal grains (Koehler & Wieser, 2013; Taylor & Schüssler, 1986), constituted the highest proportion of total grain protein in Native Millet and the second highest in Curly Mitchell Grass (Figure 5.2).

In wheat, barley, and rye, a higher ratio of prolamin to glutelin in the dough generally indicates a weaker protein structure with less elasticity and increased susceptibility to deformation of the loaf during the baking process (Girard & Awika, 2021; Janssen *et al.*, 1996). However, the relationship between the prolamin:glutelin ratio in flour and the dough characteristics of gluten-containing cereals such as wheat, barley, and rye may not be applicable to gluten-free cereals.

A review of the literature did not identify studies that examine the relationship between the prolamin:glutelin ratio and the dough properties of gluten-free cereals. Further research is essential to establish a clearer understanding of the dough rheological characteristics of the native grains in relation to the prolamin:glutelin ratio.

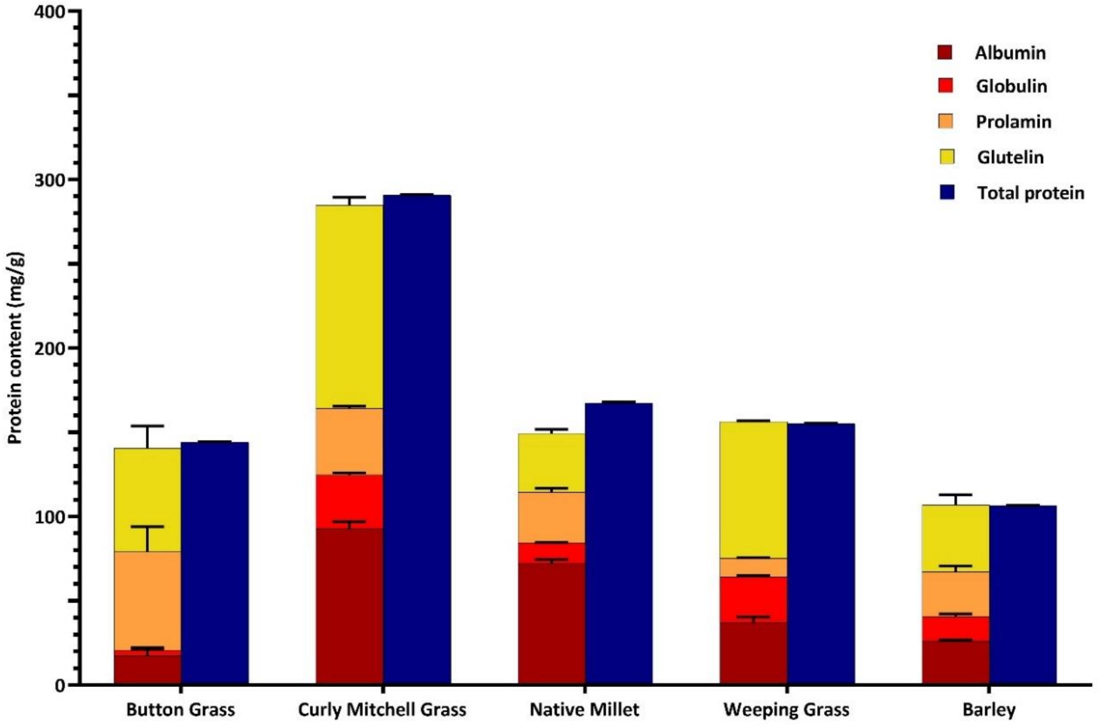

Figure 5.2. The mean concentrations of albumin, globulin, prolamin, and glutelin after semi-exhaustive extractions of wholemeal flour from Button Grass, Curly Mitchell Grass, Native Millet, Weeping Grass, and barley (control) proteins (mg/g db). These same species were also tested for total protein content, expressed as mg/g db. Error bars represent standard deviations (n = 3).

5.3.3 Total Protein Profile of Wholemeal Samples

The protein bands corresponded to a diverse range of molecular weights among the grains tested (Figure 5.3). Barley showed prominent bands at 30–75 kDa and additional faint bands below 15 kDa. Based on their electrophoretic mobilities and amino acid compositions, hordeins are divided into four groups: A (15 kDa), B (30–50 kDa), C (55–80 kDa), and D (80–90 kDa). The bands between 30–75 kDa likely correspond to B- and C-hordeins, while the faint bands below 15 kDa may represent either A-hordeins or protein degradation products (Evans & Bamforth, 2009).

In contrast, the native grains exhibited distinct protein profiles. Mitchell Grass and Button Grass, for instance, exhibited strong bands in the 10–60 kDa range. In Weeping Grass, faint bands appeared around 50 kDa, while intense bands were observed below 35 kDa. Native Millet showed a combination of protein bands in the 37–50 kDa range and below 25 kDa, which indicates a diverse protein composition with medium and low-molecular-weight fractions. The differences in protein profiles among the native grains highlight their species-specific attributes, likely shaped by evolutionary adaptations and ecological conditions. These variations could affect their suitability for different food applications, particularly for health-focused products and gluten-free alternatives.

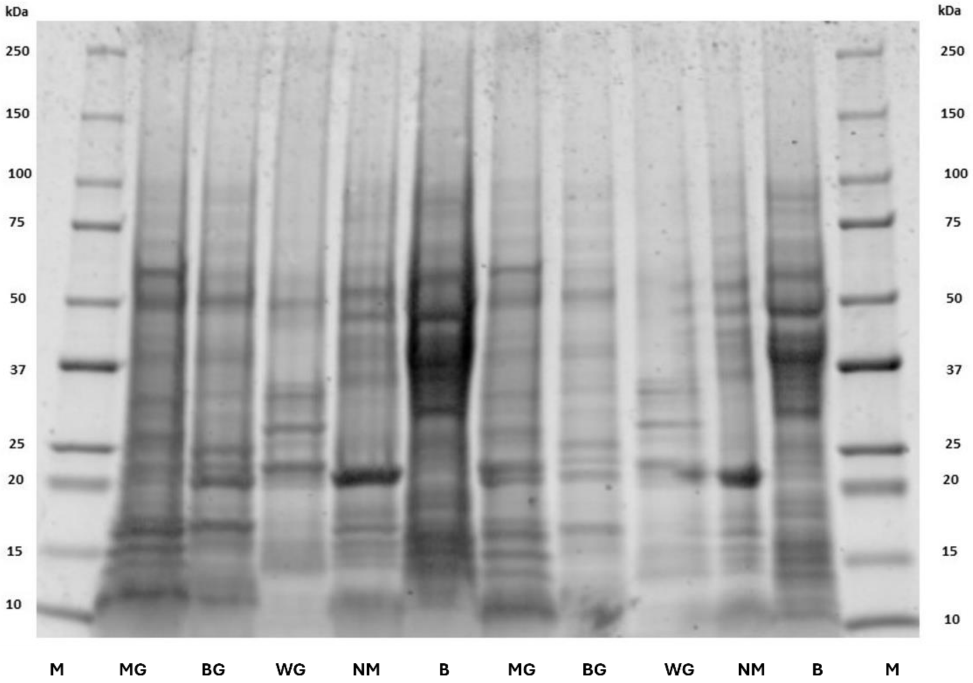

Figure 5.3. SDS-PAGE analysis of proteins from Curly Mitchell Grass (MG), Button Grass (BG), Weeping Grass (WG), Native Millet (NM), and barley (B). Proteins were extracted using Osborne fractionation. For each species, two lanes represent duplicate samples. The gel was stained with Coomassie Brilliant Blue. Lanes 1 and 12 represent Marker.

5.3.4 Protein Profile of Fractionated Proteins

Figures 5.4 and 5.5, which show SDS-PAGE profiles, reveal differences in the polypeptide composition of albumin, globulin, prolamin, and glutelin fractions across the native grains and the control. In all the native species, both high- and low-molecular-weight (HMW and LMW) proteins were observed in the albumin and globulin fractions, indicating the presence of diverse soluble protein types.

The albumin fractions of Button Grass (BG) and Curly Mitchell Grass (MG) displayed a wide distribution of bands, with molecular weights ranging from approximately 10 to 150 kDa. This reflects a complex protein profile that may include stress-responsive proteins, metabolic enzymes, and potential allergens. In Native Millet (NM) and barley (B), the albumin fractions showed prominent bands within the 10–75 kDa range. This similarity may reflect the presence of comparable protein components in these cereal grains.

All the native species displayed multiple bands in their globulin fractions, but these bands covered a narrower molecular range than those in the albumin fractions. The globulin bands were most prominent between 10 and 75 kDa, particularly in MG and NM. Based on their molecular weights and solubility characteristics, these proteins may belong to storage protein groups commonly found in the globulin fractions of cereal grains.

The prolamin fractions of BG, NM, and MG exhibited similar patterns, with major protein bands concentrated around 20 kDa. This similarity may reflect a shared composition of hydrophobic storage proteins among these native grasses.

The glutelin fractions exhibited relatively simple polypeptide profiles, characterised by bands predominantly within the low-molecular-weight range. Protein bands in the glutelin fractions appeared between 10 and 20 kDa in BG, NM, and MG, while those in WG were limited to a narrower range of 10 to 15 kDa.

In conclusion, the SDS-PAGE profiles reveal both unique and shared proteins across the native grasses and control barley. Differences in band intensity and distribution among Osborne fractions suggest variation in protein composition likely due to genetic divergence.

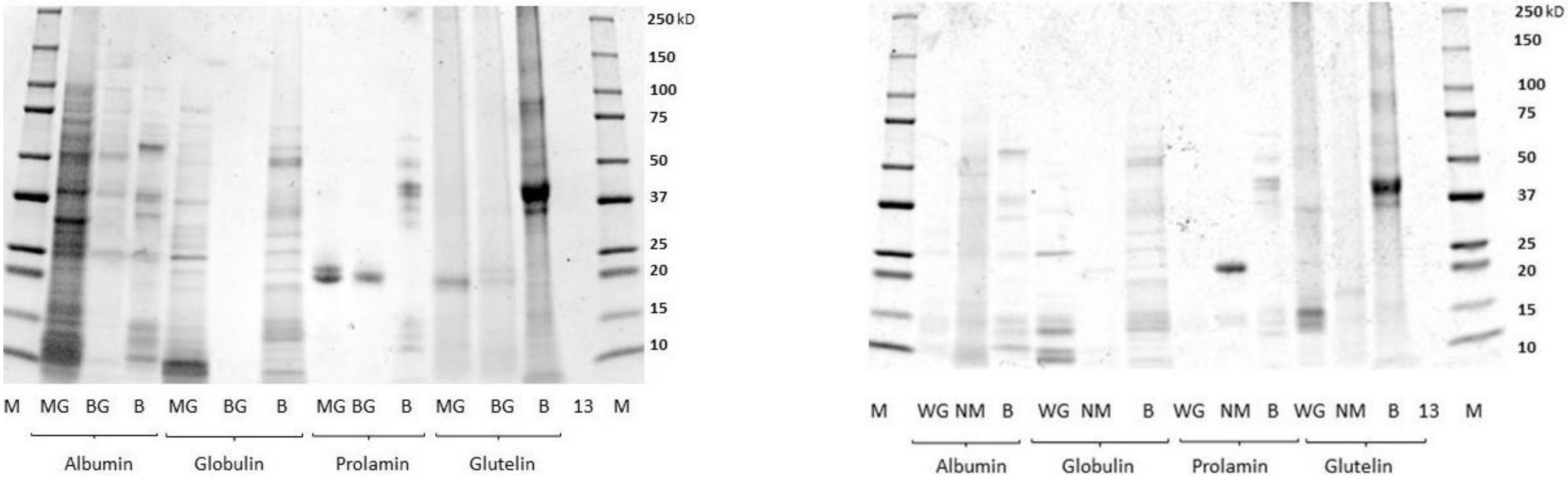

Figure 5.4. SDS-PAGE analysis showing the polypeptide profiles of albumin, globulin, prolamin, and glutelin fractions in Button Grass (BG), Curly Mitchell Grass (MG), Native Millet (NM), Weeping Grass (WG), and barley (B). The gels were stained with Coomassie Brilliant Blue. Lanes 13 and M represent blank and Marker, respectively.

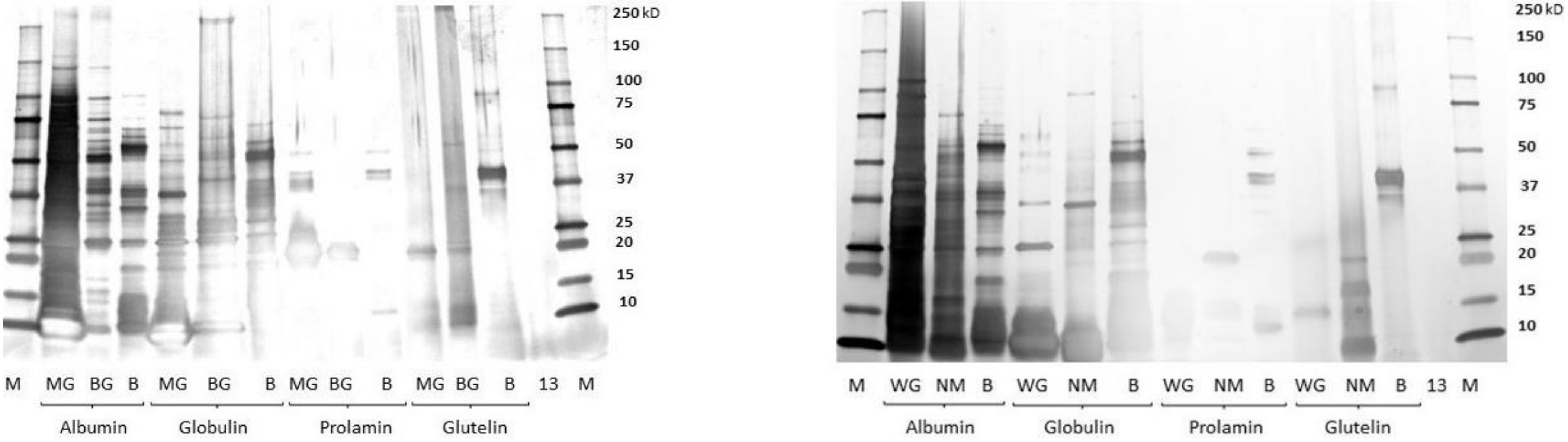

Figure 5.5. SDS-PAGE analysis showing the polypeptide profiles of albumin, globulin, prolamin, and glutelin fractions in Button Grass (BG), Curly Mitchell Grass (MG), Native Millet (NM), Weeping Grass (WG), and barley (B). The gels were stained with silver nitrate. Lanes 13 and M represent blank and Marker, respectively.

5.4 Conclusion

This chapter presented characterisation of the protein composition of four Australian native grains—Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass—using a combination of gluten screening, Osborne fractionation, and SDS-PAGE analysis. The gluten detection assays confirmed the absence of gluten in all four species, supporting their potential application in gluten-free food products and highlighting their relevance for individuals with coeliac disease or gluten intolerance.

Osborne fractionation revealed substantial variation among the native grain species in the distribution of major protein solubility classes (albumins, globulins, prolamins, and glutelins). In most of the native grains, glutelin was the dominant protein fraction, a pattern also observed in cereals that have high levels of storage proteins. Native Millet and Weeping Grass showed contrasting protein profiles; Native Millet was distinguished by a higher proportion of albumins, whereas Weeping Grass had the highest glutelin content among the species tested. In other cereals, glutelins are commonly associated with storage or structural roles (Shewry & Halford, 2002), suggesting this fraction may contribute similarly in Weeping Grass.

SDS-PAGE analysis further elucidated the molecular diversity of the protein fractions. A wide range of molecular weights in the albumin and globulin fractions revealed a complex mixture of functional proteins, likely including enzymes. In contrast, the prolamin and glutelin fractions primarily consisted of polypeptides with low molecular weights, which may relate to storage protein characteristics typically observed in cereal grains. The results indicate that each native grain has a unique protein composition, which could affect functional properties in food systems, including solubility, digestibility, and behaviour during processing.

Together, these results underscore the biochemical diversity among Australian native grains and demonstrate their potential as novel, gluten-free protein sources in both traditional and modern food applications. Proteomic analysis of the wholemeal flour of the native grains is described in Chapter 6, which complements the results of this chapter.

Chapter 6

Proteomic characterisation of Australian native grains

6.1 Introduction

Proteomics represents an important analytical approach in plant science for investigating the complete protein profile of organs (such as seeds), tissues, or cells. When applied to grains, the results of proteomics experiments enhance our understanding of grain development, including nutrient accumulation, and the implications of the protein profile for human nutrition. Liquid chromatography–mass spectrometry (LC-MS), which is the standard proteomics approach in contemporary laboratories (having long superseded 2D-gel electrophoresis), offers high sensitivity, accuracy, and the ability to detect large numbers of proteins from complex grain samples (Alves *et al.*, 2019; Labuschagne, 2018).

Domesticated cereals have been well studied in terms of their gene function, protein composition, and metabolic pathways, due to the availability of complete genomes, masses of transcriptomic data, and corresponding virtual proteome databases. However, to date, no complete reference genomes are publicly available for the four Australian native grasses analysed in this study. Thus, due to this limitation, the experiments reported in this chapter represent pioneering attempts to understand the grain proteomes of these species.

Dactyloctenium radulans (Button Grass) has been included in genome-related initiatives, such as a Bioplatforms Australia project (NCBI: PRJEB49212) and a NCBI RefSeq genome project (NCBI: PRJNA550819). However, no genome sequence has been released.

Astrebla lappacea (Curly Mitchell Grass) is similarly part of the Bioplatforms initiative (NCBI: PRJEB49212), but no species-specific genomic data are currently accessible.

Panicum decompositum (Native Millet) has been included in the PILBseq project (NCBI: PRJNA522689), which aimed to generate plastid and ribosomal DNA sequences from Australian herbarium specimens. Although the species is part of this project, no complete genome sequence is currently available. Only limited nucleotide and protein sequences have been made publicly accessible in online databases (NCBI:txid206018). The species is also listed in the Bioplatforms Australia initiative (NCBI: PRJEB49212), but no nuclear genome sequence is currently accessible.

Microlaena stipoides (Weeping Grass) is also an object of several genome-related efforts, including projects led by Bioplatforms Australia (NCBI: PRJEB49212) and Southern Cross University (NCBI: PRJEB4624; PRJNA221130), aimed at advancing its genetic characterisation. However, to date, no complete genome sequence for this species has been made publicly available.

Due to the absence of publicly available full genomes for the four species studied in this PhD research, protein identification was conducted using the Swiss-Prot Green Plants database. This database provided the broadest possible coverage of plant proteins rather than species-specific data. The Swiss-Prot Green Plants database includes over 570,000 manually curated protein entries from more than 14,000 species (UniProt Consortium, 2025).

LC-MS proteomics of grains of species with unsequenced genomes allows putative identification of a wide range of proteins, including enzymes, structural proteins, stress-related proteins, chaperones, and storage proteins. Many of these proteins play key roles in seed development and may affect both the nutritional value and food processing characteristics of the grains/flour. Proteomic results from species without sequenced genomes can help identify important proteins for future gene discovery and genome assembly (Renuse *et al.*, 2012).

The protein profiles of four Australian native grass species are presented in this chapter, based on bottom-up LC-MS proteomics, a method that involves enzymatically digesting proteins into peptides prior to mass spectrometry analysis. All proteins were extracted from wholemeal flour using a consistent preparation method. Protein identification was based on matches to a reference plant protein database, which allowed classification by type and relative abundance. Although genome sequences are unavailable for these species, the results provide a useful overview of their grain protein composition and establish a foundation for future research.

6.2 Materials and Methods

6.2.1 Plant Materials

The plant materials used in this study were the same as those described in Section 2.2.1 of Chapter 2. Barley, one of the reference grains described in that section, was used as the control in this study.

6.2.2 Flour Preparation

Approximately 25 mg of each grain sample was weighed and finely ground using a mortar and pestle under liquid nitrogen to facilitate cell disruption. The resulting flour was immediately transferred to low-binding 2-mL Eppendorf tubes and stored at −30 °C until further use.

6.2.3 Protein Extraction and Digestion

Protein extraction and digestion were carried out using a modified version of the SDC-FASP (sodium deoxycholate–filter-aided sample preparation) method, adapted from the protocol described by Wang *et al.* (2018). The protocol was optimised for use with flour samples derived from native grains by reducing the sample input from 500 mg to 25 mg, and by substituting Tris-(2-carboxyethyl)phosphine (TCEP) for dithiothreitol as the reducing agent and chloroacetamide (CAA) for iodoacetamide as the alkylating agent.

6.2.3.1 Reagents and Materials

The following reagents were used for protein extraction and digestion.

- Protein extraction buffer, composed of:
  - 10 mM TCEP, used as a reducing agent
  - 2% (w/v) sodium deoxycholate (SDC), a detergent for solubilising proteins
  - 1% (w/v) polyvinylpolypyrrolidone (PVPP), for binding phenolic compounds
  - 0.3 M triethylammonium bicarbonate (TEAB), pH 8.5
  - Protease inhibitor cocktail (e.g., Omplete™, EDTA-free), 1 tablet per 50 mL
  - Phosphatase inhibitor cocktail (e.g., PhosSTOP™), 1 tablet per 10 mL.

Additional reagents and materials used in the protocol are listed below:

- 1% SDC in 0.1 M TEAB (pH 8.5), used for washing and sample clean-up
- CAA solution, containing 40 mM CAA in 1% SDC, for alkylation
- Trypsin solution, prepared at 0.04 µg/µL in 1% SDC, for enzymatic digestion
- Filter device: Microcon spin filters with a 10–30 kDa molecular weight cutoff (Vivaspin® 500, Sartorius, Göttingen, Germany) were used both before and after enzymatic digestion. Before digestion, the filters served to concentrate the protein extract, remove low-molecular-weight contaminants, and facilitate buffer exchange. Following digestion, they allowed the separation of peptides from undigested proteins and other high-molecular-weight components, ensuring that only low-molecular-weight peptides were recovered for LC-MS analysis.

6.2.3.2 Sample Preparation and Protein Extraction

To each tube containing approximately 25 mg of sample flour was added 0.5 mL of extraction buffer, and the mixture was homogenised on ice with a pipette tip for about 3 min. An additional 0.3 mL of buffer was used to rinse residual material from the mortar, and the rinse was subsequently added to the tube. Samples were vortexed for 10 s, incubated at 80 °C for 10 min to denature proteins, and then cooled on ice.

Each sample underwent sonication using a probe sonicator with two 30-s bursts, separated by 30 s on ice. Following sonication, samples were shaken gently at RT for 30 min. The homogenised samples were centrifuged at 10,000 × *g* for 10 min, and the supernatants were transferred to new tubes. A second centrifugation at 20,000 × *g* for 15 min was performed to remove remaining insoluble particles from the sample. Protein concentrations were determined using the Qubit™ Protein Assay Kit (Thermo Fisher Scientific), following the manufacturer's instructions.

6.2.3.3 Protein Clean-up and Alkylation

An aliquot of 100 µg of total protein was loaded into a Microcon spin filter (10–30 kDa cutoff) and washed with 200 µL of 1% SDC solution by centrifugation at 10,000 × *g* for 15 min. The flow-through was discarded, and the retained proteins on the membrane were alkylated by adding 100 µL of CAA solution. The mixture was incubated in the dark at RT for 30 min, followed by centrifugation at 10,000 × *g* for 15 min.

Proteins were then washed twice with 100 µL of 1% SDC solution, each followed by centrifugation at 10,000 × *g* for 15 min to remove excess reagents and contaminants.

6.2.3.4 Trypsin Digestion

Trypsin digestion was performed using Sequencing Grade Modified Trypsin (Promega, Catalog Number: V5111), in which lysine residues have been modified by reductive methylation to enhance specificity and stability. A total of 50 µL of trypsin solution was added to the spin filter (enzyme-to-protein ratio 1:50), followed by a 6-h incubation at 37 °C. Trypsin cleaves peptide bonds at the carboxyl side of lysine and arginine residues, except when followed by proline, and is widely recognised as suitable for peptide generation in mass spectrometry analysis (Olsen *et al.*, 2004).

The spin filter was then centrifuged at 10,000 × *g* for 15 min, and the resulting flow-through containing the released peptides was collected. An additional 50 µL of 1% SDC solution was added to the filter to maximise peptide recovery. The filter was centrifuged again under the same conditions, and the second flow-through was collected and combined with the first.

6.2.3.5 Peptide Clean-up and LC-MS Analysis

Peptides were purified using an ethyl acetate phase separation method. DMSO was added to the peptide solution to reach a final concentration of 10% (v/v), followed by the addition of ethyl acetate at a 3:1 ratio and trifluoroacetic acid (TFA) to reach a final concentration of 0.5%. After mixing the samples at 1,000 rpm for 5 min at 10 °C, they were briefly vortexed and centrifuged at 15,000 × *g* for 10 min at the same temperature.

The lower aqueous phase was then carefully collected, with approximately 90% transferred to a fresh tube. Then, 300 µL of ethyl acetate was added for a second wash to enhance peptide purification. The mixture was again mixed, vortexed, and centrifuged under the same conditions. The upper organic layer was discarded, and the resulting peptide solution was stored.

Following phase separation, peptides were further purified using SDB-RPS StageTips packed with two layers of SDB-RPS discs (Sigma-Aldrich, Cat. No. 66886-U). The tips were mounted in a 3D-printed holder above a 96-well polypropylene waste plate. Tips were first wet with 100 µL of 100% acetonitrile (ACN) and centrifuged at 1,000 × *g* for 1 min. This was followed by equilibration with 100 µL of 30% methanol containing 1% TFA, followed by 100 µL of 0.2% TFA.

Both equilibration solutions—30% methanol with 1% TFA and 0.2% TFA—were centrifuged through the tips at 1,000 × *g* for 3 min. The peptide samples were loaded onto the tips and then centrifuged under the same conditions. The bound peptides were washed in sequence with 100 µL of 99% ethyl acetate containing 1% TFA (twice), followed by 100 µL of 0.2% TFA.

Each wash step was followed by centrifugation at 1,000 × *g* for 3 min. Bound peptides were extracted by adding 100 µL of 5% ammonium hydroxide in 80% ACN to each tip, followed by centrifugation at 1,000 × *g* for 5 min. A Genevac evaporator (45 °C, NH_3-H_2O mode) was used to dry the peptides, which were then resuspended in 40 µL of 3% ACN containing 0.1% formic acid. The samples were then stored at 4 °C until LC-MS analysis was carried out using a Q Exactive HFX mass spectrometer.

6.2.3.6 Protein Identification and Functional Classification

Raw LC–MS/MS data were analysed using Proteome Discoverer version 3.1.1.93 (Thermo Fisher Scientific). Proteins were identified using the Sequest HT search engine against the Swiss-Prot Green Plants database.

A 1% false discovery rate (FDR) was applied at both the peptide and protein levels using a target-decoy approach, which estimates the rate of false positives by searching the spectra against a combined database of real (target) and reversed or randomised (decoy) protein sequences.

The top 100 most abundant proteins, ranked by peptide-spectrum matches (PSMs), were listed for each species and are presented in Appendix Tables 6.1–6.5.

6.3 Results and Discussion

This section presents the major proteins identified in the four native Australian grains. It outlines differences in the annotated source species of matched proteins and emphasises key categories such as stress-related, storage, and database-assigned species-specific proteins. Relevant comparisons to domesticated cereals are included to highlight likely nutritional and functional implications of the identified proteins.

6.3.1 Source Species Contribution to Identified Proteins

The number of proteins identified based on peptide matches to known sequences from other species differed among the native grain species (Figure 6.1). For example, Weeping Grass had the highest number of protein matches with *Oryza sativa*, which aligns with its close taxonomic relationship to rice (Kasem *et al.*, 2011b; Shapter *et al.*, 2013).

Button Grass, Native Millet, and Curly Mitchell Grass, by contrast, showed a broader diversity of protein matches. These included not only species from the *Poaceae* family but also a range of other plants, most of which are eudicots, such as *Arabidopsis thaliana*, *Brassica napus*, *Solanum lycopersicum*, *Petunia hybrida*, *Spinacia oleracea*, *Gossypium hirsutum*, *Antirrhinum majus*, and *Nicotiana tabacum*. The presence of protein matches from such a wide taxonomic range—including eudicot species, which are phylogenetically distinct from grasses, as well as basal angiosperms like *Magnolia liliiflora*—may reflect both evolutionary distance and differences in protein annotation across reference genomes.

Although the sample was derived from *Hordeum vulgare* (barley), not all of the top 20 proteins matched to *Hordeum vulgare* entries in the Swiss-Prot database (Figure 6.1). This may be due to the similarity of peptide sequences among related species and the limited number of *Hordeum*-specific entries in the Swiss-Prot database. As a result, some peptides were matched to proteins from other species (Schnoes *et al.*, 2013).

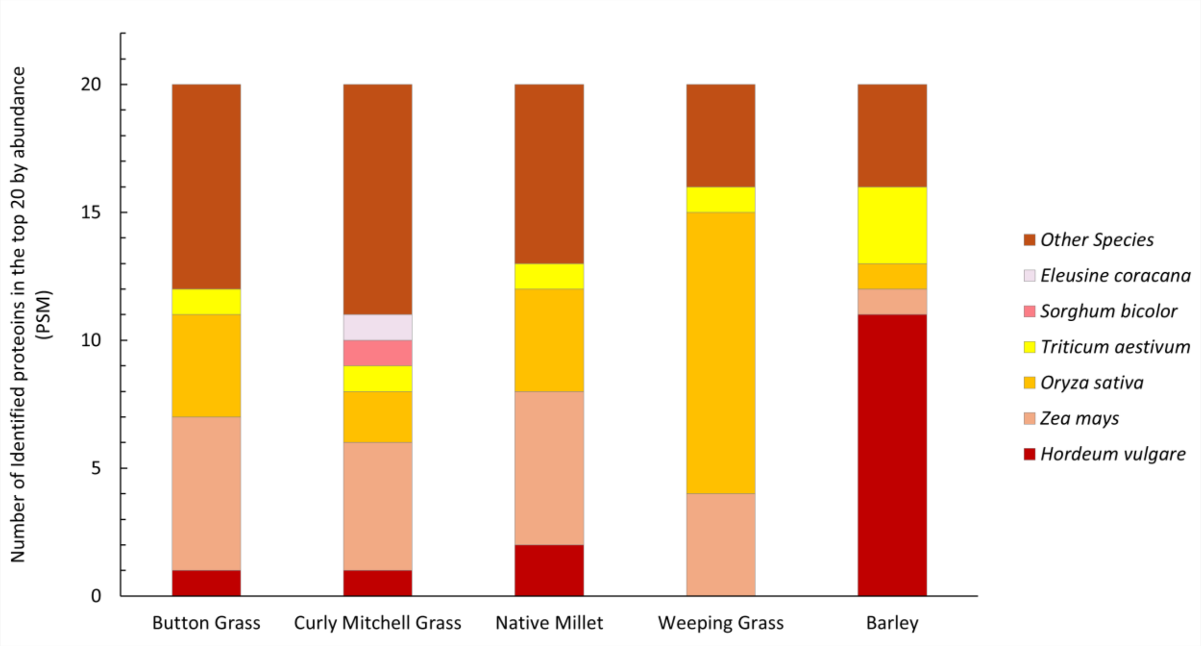

Figure 6.1. Number of the top 20 proteins—ranked by peptide spectrum matches (PSMs)—in each grain species that matched known proteins from various plant sources, based on sequence similarity to entries in the Swiss-Prot Green Plants database. Matches to *Poaceae* species (including *Hordeum vulgare* (barley), *Zea mays* (maize), *Oryza sativa* (rice), *Triticum aestivum* (bread wheat), *Sorghum bicolor* (sorghum), and *Eleusine coracana* (finger millet)) are shown individually, while matches to non-*Poaceae* species are grouped under “Other Species”.

6.3.2 Stress-Responsive Proteins

6.3.2.1 Heat Shock Proteins (HSPs) and Molecular Chaperones

Heat shock proteins (HSPs) are a group of molecular chaperones that help protect cells by assisting in protein folding, preventing misfolding, and stabilising proteins during stress. Among these, the 70 kDa family (HSP70s) plays a particularly important role in plant stress responses by promoting correct protein folding, preventing aggregation, and maintaining protein stability under adverse conditions (Khan & Shahwar, 2020; Latijnhouwers *et al.*, 2010).

In this study, the native grains showed higher HSP70 diversity than barley among the top 20 most abundant proteins (Table 6.1). Curly Mitchell Grass expressed the highest number of

HSPs, including several proteins known to assist in protein folding during stress (Babu *et al.*, 2009; Flores-Pérez & Jarvis, 2013).

Native Millet expressed four HSPs: Heat shock 70 kDa protein 4, Chloroplast envelope membrane 70 kDa heat shock-related protein, Heat shock 70 kDa protein 6, chloroplastic—commonly linked to stress adaptation in chloroplasts (Ding *et al.*, 2022)—and Heat shock 70 kDa protein 18.

Weeping Grass expressed both general HSP70s (e.g., Heat shock 70 kDa protein 2 and Heat shock 70 kDa protein) and two smaller forms—26.7 kDa heat shock protein, chloroplastic, and 16.9 kDa class I heat shock protein 1. Hsp16.9A participates in cytoplasmic protein complexes that support thermotolerance, while Hsp26.7 contributes to chloroplast protection under high-temperature conditions (Chen *et al.*, 2014).

The only three HSPs found in the top 20 proteins in barley were also present in Curly Mitchell Grass. The broader range of HSPs observed in native grains suggests adaptation to various environmental stresses, including heat, drought, and oxidative stress, and highlights their value as genetic resources for the development of stress-resilient crops (Rauch *et al.*, 2016; Staacke *et al.*, 2025).

Table 6.1. Heat shock proteins (HSPs) and molecular chaperones identified among the top 20 most abundant proteins—ranked by peptide spectrum matches (PSMs)—in native Australian grains and barley, based on LC–MS/MS analysis and sequence match to the Swiss-Prot Green Plants database. BG stands for Button Grass, MG for Curly Mitchell Grass, NM for Native Millet, and WG for Weeping Grass.

Protein Description	**Matched Species (from Database)**	**Detected in**	**UniProt ID**
Heat shock 70 kDa protein 2	*Arabidopsis thaliana*	BG, MG, WG	P22954
Heat shock 70 kDa protein 4	*Arabidopsis thaliana*	MG, NM, Barley	Q9LHA8
Chloroplast envelope membrane 70 kDa heat shock-related protein	*Spinacia oleracea*	MG, NM	P29357
Heat shock cognate 70 kDa protein	*Petunia hybrida*	MG, Barley	P09189
Heat shock cognate 70 kDa protein 2	*Solanum lycopersicum*	MG, Barley	P27322
Heat shock 70 kDa protein BIP2	*Arabidopsis thaliana*	MG	Q39043
Heat shock 70 kDa protein BIP1	*Oryza sativa*	MG	Q6Z7B0
Heat shock 70 kDa protein 6, chloroplastic	*Arabidopsis thaliana*	NM	Q9STW6
Heat shock 70 kDa protein 18	*Arabidopsis thaliana*	NM	Q9C7X7
Heat shock 70 kDa protein	*Zea mays*	WG	P11143
26.7 kDa heat shock protein, chloroplastic	*Oryza sativa*	WG	Q10P60
16.9 kDa class I heat shock protein 1	*Oryza sativa*	WG	P27777

6.3.2.2 Other Stress-Protective Proteins

Late embryogenesis abundant (LEA) proteins—known for protecting seeds under environmental stress—were abundant in multiple species among the stress-related proteins identified in the studied grains (Table 6.2). The LEA group 3 protein was detected in Button Grass, Curly Mitchell Grass, and Native Millet, while LEA protein B19.1A was present in the same three grains as well as barley. Another LEA group 3 protein was found in Curly Mitchell Grass and barley, while LEA protein EMB564 was detected only in Native Millet.

In addition, an ABA-inducible protein (PHV A1), which may be involved in responses to drought or seed maturation (Nakashima & Yamaguchi-Shinozaki, 2013), was present in Native Millet and barley. Barley also featured three other stress-associated proteins: Em protein CS41, LEA protein B19.4, and LEA protein B19.3, all of which have been reported in studies of stress responses (Chen *et al.*, 2022; Stacy *et al.*, 1995).

The results showed that stress-protective proteins were more widespread in some native grain samples than others. While this may suggest species-level differences in stress-related protein expression, it is important to note that the grains were not collected under uniform environmental conditions, and these differences could also reflect variation in environmental exposure.

Table 6.2. Stress-protective proteins identified among the top 20 most abundant proteins—ranked by peptide spectrum matches (PSMs)—in native Australian grains and barley, based on LC–MS/MS analysis and sequence matches to the Swiss-Prot Green Plants database. BG stands for Button Grass, MG for Curly Mitchell Grass, NM for Native Millet, and WG for Weeping Grass.

Protein Name	Matched Species (from Database)	Detected in	UniProt ID
LEA protein, group 3	*Zea mays*	BG, MG, NM	Q42376
LEA protein B19.1A	*Hordeum vulgare*	BG, MG, NM, Barley	Q05190
LEA protein, group 3	*Triticum aestivum*	MG, Barley	Q03968
ABA-inducible protein PHV A1	*Hordeum vulgare*	NM, Barley	P14928
LEA protein EMB564	*Zea mays*	NM	P46517
Em protein CS41	*Triticum aestivum*	Barley	P22701
LEA protein B19.4	*Hordeum vulgare*	Barley	Q05191
LEA protein B19.3	*Hordeum vulgare*	Barley	Q02400

6.3.3 Storage Proteins

Only one hordein protein was identified among the top 20 in barley. The limited detection of hordein peptides may result from the use of trypsin in the proteomic workflow, since hordein is low in lysine and arginine and high in proline and glutamine, which restricts cleavage efficiency (Colgrave *et al.*, 2017; Řehulková *et al.*, 2009).

Storage proteins were among the most abundant proteins in the native grains, except for Button Grass (Table 6.3). Weeping Grass had the highest number, with four distinct storage proteins identified: seed storage globulin type A3, glutelin types A2 and B1, and seed storage globulin 1 protein. These proteins typically contribute to nutrient storage and seed development, and their functions are comparable to those of storage proteins in domesticated cereals (Shewry & Halford, 2002).

Mitchell Grass and Native Millet each contained one globulin-type seed storage protein, but the specific proteins differed between the two species.

The presence of these proteins in native grains supports their nutritional relevance and indicates potential for use in food applications comparable to conventional cereals.

Table 6.3. Storage proteins identified among the top 20 most abundant proteins—ranked by peptide spectrum matches (PSMs)—in native Australian grains and barley, based on LC–MS/MS analysis and sequence match to the Swiss-Prot Green Plants database. BG stands for Button Grass, MG for Curly Mitchell Grass, NM for Native Millet, and WG for Weeping Grass.

Protein Name	**Matched Species (from Database)**	**Detected in**	**UniProt ID**
Seed storage globulin	*Brassica napus*	MG	P33525
Globulin-like storage protein	*Oryza sativa*	NM	Q75GX9
Glutelin type-B1	*Oryza sativa*	WG	P14323
Glutelin type-A2	*Oryza sativa*	WG	P07730
Globulin type-A3	*Oryza sativa*	WG	Q09151
Seed storage Globulin 1	*Avena sativa*	WG	P12615
Hordein Storage	*Hordeum vulgare*	Barley	P06471

6.3.4 Distinctive Proteins Detected in Weeping Grass and Native Millet

Based on the list of identified proteins in Weeping Grass, there is a strong similarity with proteins previously identified in rice (*Oryza sativa*), reflecting their close evolutionary relationship within the subfamily *Oryzoideae* (Kasem *et al.*, 2011a; Shapter *et al.*, 2013). Among the top 20 proteins detected, 11 matched entries from *Oryza sativa*, along with one from the closely related African rice species *Oryza glaberrima* (Table 6.4).

The identification of 12S seed storage globulin 1, which is known to improve foaming capacity (Janssen *et al.*, 2025; Nieto-Nieto *et al.*, 2014), may explain the high foaming capacity (50%) observed in Weeping Grass under alkaline conditions (Chapter 3).

The presence of Oleosin 18 kDa in both Native Millet and *Oryza sativa* may be associated with the high lipid content in Native Millet seeds (8.0 ± 0.6% db; Chapter 3), given its important role in lipid accumulation in rice (Chen *et al.*, 2019; Wu *et al.*, 2010).

Table 6.4. Proteins identified in *Microlaena stipoides* (Weeping Grass) that matched sequences from *Oryza sativa* (rice), among the top 20 most abundant in Weeping Grass.

Shared Proteins Between *Oryza sativa* (rice) and *Microlaena stipoides* (Weeping Grass)	**UniProt ID**
Divinyl chlorophyllide a 8-vinyl-reductase, chloroplastic	D5L1S4
Granule-bound starch synthase 1, chloroplastic/amyloplastic	Q42968
Glutelin type-B 1	P14323
Glutelin type-A 2	P07730
Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	Q7FAH2
Glutelin type-A 3	Q09151
Ketol-acid reductoisomerase, chloroplastic	Q65XK0
1-Cys peroxiredoxin A	P0C5C9
26.7 kDa heat shock protein, chloroplastic)	Q10P60
Chaperone protein ClpB2, chloroplastic	Q75GT3
ATP synthase subunit alpha, mitochondrial	P0C520
16.9 kDa class I heat shock protein 1	P27777

6.4 Conclusion

This chapter presents the first proteomic characterisation of Button Grass (*Dactyloctenium radulans*), Curly Mitchell Grass (*Astrebla lappacea*), Native Millet (*Panicum decompositum*), and Weeping Grass (*Microlaena stipoides*) using bottom-up LC–MS/MS. Protein identification with reference to the Swiss-Prot Green Plants database enabled the matching to a wide range of proteins involved in stress response, nutrient storage, and seed development, despite the absence of complete reference genomes.

Notable differences were observed in the diversity and composition of proteins among the native grains. The strong match between Weeping Grass and rice proteins reflects its close evolutionary relationship with *Oryza sativa* and points to potential similarities in protein function.

The presence of stress-associated proteins such as HSPs and LEA proteins in native species—in most cases at greater diversity than in barley—indicates strong adaptive mechanisms that may contribute to the resilience of grain development in challenging environments. These stress-related proteins may contribute to the ecological success of native grains and could help inform future efforts to improve stress tolerance in commercial cereals.

Moreover, the presence of key storage proteins—such as globulins and glutelins—highlights the nutritional value of native grains, as these proteins are rich in essential amino acids and play an important role in determining the protein quality and content of the grain.

In conclusion, the proteomic analysis described in this chapter offers the first detailed overview of the protein composition in Australian native grains and highlights their potential for agricultural and nutritional innovation.

Chapter 7

General discussion and future directions

7.1 Conclusion

7.1.1 General Overview

Worldwide agricultural priorities are shifting notably in response to global challenges like climate change, biodiversity loss, and food insecurity. These pressures encourage exploration of resilient and sustainable crop systems capable of adapting to increasingly variable environmental conditions. In this context, underutilised native crops are being explored not only for their cultural importance, but for their ecological adaptability, as potential contributors to economically and environmentally sustainable agriculture. Beyond agronomic considerations, these native species often contain nutritional components and bioactive compounds that may contribute to improved human health, suggesting their potential role in functional and specialty food markets (Farooq *et al.*, 2023; Jenkins *et al.*, 2023; Kaur *et al.*, 2025).

This thesis examined four Australian native grasses—*Dactyloctenium radulans* (Button Grass), *Astrebla lappacea* (Curly Mitchell Grass), *Panicum decompositum* (Native Millet), and *Microlaena stipoides* (Weeping Grass)—to explore their potential role in broadening the range of grains used in food production. These species were selected based on their production potential within their natural range, practical threshability, and commercial promise.

A comparative analysis was conducted between these native grains and wheat, barley, and sorghum, the most economically important cereals in Australia. The study examined grain microstructure and morphology to characterise physical attributes that could influence processing and product quality. Proximate composition and key functional properties were evaluated to assess nutritional and technological potential. Carbohydrate and protein profiles were investigated to understand macronutrient composition and reveal features relevant to digestibility, processing behaviour, and potential health benefits. Advanced proteomic techniques were employed to boost our understanding of the identity and potential functional roles of the grain proteins, which have implications for food processing and potential health benefits.

7.1.2 Summary of Key Findings

7.1.2.1 Morphological and Structural Characteristics

Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass exhibited distinctive morphological traits (detailed in Chapter 2) that differentiate them from domesticated cereals. Key characteristics comprised variations in kernel size, shape, and density, as well as distinctive embryo proportions and patterns of starch and protein distribution within the grain. These structural features are expected to influence nutritional quality and affect processing behaviour, including threshing and milling efficiency (Abedi *et al.*, 2023).

While some traits, such as small and dense kernels or a large embryo size in relation to the whole grain, present challenges for mechanised processing and product stability; they also offer potential functional advantages for specific food applications. Small and dense kernels may require specialised milling techniques but have the potential to yield flours with distinctive textural properties, such as finer particle size, enhanced moisture retention, altered crumb structure, and unique mouthfeel characteristics. These qualities make such flours particularly suitable for niche applications in specialty baked goods and innovative snack products. A relatively larger embryo can also improve nutrient density in wholegrain products, as the embryo contains higher levels of proteins, lipids, vitamins, minerals, and antioxidants compared to the starchy endosperm, enriching the nutritional profile of the grain; however, the higher lipid content may also reduce shelf life due to increased susceptibility to rancidity (Ma *et al.*, 2020; Rosa-Sibakov *et al.*, 2015).

Microscopic localisation of key macronutrients—such as starch, beta-glucan, and protein—in the grains offers insights into their potential digestibility, influenced by factors like encapsulation within cell walls and accessibility to digestive enzymes, which in turn affect their health benefits, including glycaemic response and gut health (Singh *et al.*, 2010). The morphological insights developed for these four grains form a foundational understanding to guide future research on processing optimisation and product development.

7.1.2.2 Nutritional Composition, Carbohydrate Profile, and Protein Profiles

All four native grains tested negative for gluten, confirming their gluten-free status. They also showed nutritionally relevant traits, such as moderate to high protein levels and elevated dietary fibre content compared to commercial grains, as detailed in Chapters 3 and 4. Some species stood out for high levels of resistant starch, beta-glucan, and amylose levels, which may enhance blood sugar regulation and digestive health when these grains are incorporated into functional food products (Binou *et al.*, 2021; Li *et al.*, 2024). This potential is supported by Pour *et al*. (2024), who reported that incorporating 10% native grain flour (the specific native grains used were not identified) into wheat-based products significantly lowered postprandial blood glucose and glycaemic index.

The native grains studied exhibited different protein profiles with some variation in key protein fractions compared to domesticated cereals. Osborne fractionation revealed differences in the relative abundance of these fractions among species. This variation likely reflects differences in protein structure and solubility, both of which influence protein behaviour during food processing and digestion. Glutelin, the predominant storage protein in all the grains except Native Millet, plays a key role in dough strength and elasticity.

Proteomic analysis revealed a diverse range of abundant proteins associated with stress response and nutrient storage, such as heat shock proteins and storage globulins, which likely improve the environmental resilience of native grains during development and their potential functionalities in gluten-free and specialty food products.

7.1.2.3 Functional Properties

The carbohydrate and protein profiles of the native grains—including resistant starch, beta-glucan, dietary fibre, and functional proteins—may also contribute functional benefits, such as improved texture, moisture retention, and shelf life, when used in fibre-enriched food formulations (Mohebbi *et al.*, 2018). The functional properties of wholegrain flours from the native grains suggest promising applications in niche food products. Weeping Grass had the highest water absorption capacity, while it also showed the highest oil absorption capacity, both of which are important for moisture retention and mouthfeel in baked and extruded products (Van Toan & Tran, 2012). Curly Mitchell Grass exhibited the highest bulk density among the native grains, indicating a denser flour structure suitable for compact food formulations. At pH 10, Curly Mitchell Grass and Weeping Grass demonstrated measurable foaming capacity, with Curly Mitchell Grass showing the strongest performance among the native grains.

Modifications such as pH adjustment through alkalisation or enzymatic treatment may improve the functional properties of the flour by increasing protein solubility and surface activity. These changes could expand their use in food products and help improve consumer acceptance of native grain-based foods.

7.1.3 Limitations

The following limitations are important to consider when evaluating the outcomes of this PhD research:

- Environmental variation was not considered in this study, as all samples originated from specific locations with limited information available on growing conditions. Environmental factors—including rainfall, soil type, temperature, and duration of light exposure—can considerably influence grain composition and functional traits (Endalamaw *et al.*, 2025; Tester & Karkalas, 2001). Therefore, evaluating genotype × environment interactions is an important area for future research, since it remains unclear whether the biochemical and functional profiles uncovered in this study are consistently exhibited under different agronomic conditions and growing seasons.
- The absence of full genome sequences negated the ability to identify species-specific proteins during proteomic analysis, reducing the precision and depth of protein characterisation for the native grains studied. This limitation underscores the need for comprehensive genomic resources to advance proteomic investigations and enhance protein annotation accuracy.
- Building on the functional and compositional findings of this study, future research could include rheological and sensory evaluations to further explore how native grain flours perform in specific food applications and how consumers perceive these products. Such studies would complement the current work by providing deeper insight into processing behaviour and guiding product formulation strategies. These additional evaluations will support the broader goal of facilitating commercial development and market acceptance of foods incorporating native grains. In the current study, such evaluations could not be undertaken due to limited flour availability and the larger sample quantities required for these tests.
- The behaviour of native grain flour during industrial processing may not be fully reflected by functional tests conducted under controlled laboratory conditions. Consequently, applying these findings to large-scale food production requires further validation under manufacturing conditions.

7.1.4 Integrating Native Grains into Existing Cropping Systems

Warm-season native grasses and domesticated winter cereals are seasonally and biologically complementary, offering potential for integration into existing cropping systems. However, successful implementation would require major adjustments to current management practices, as methods such as pasture cropping are not yet widely adopted or optimised. Instead, native grains could be integrated into current agricultural rotations by occupying seasonal gaps—for example, by growing warm-season native grasses after winter cereals. This approach would make use of fallow periods without displacing existing crops.

The ability of native grasses to grow with minimal inputs also reduces competition for resources such as water and fertiliser, supporting more efficient land use within established farming systems. In mixed systems, these grasses may stay in the field during fallow periods or off-seasons. To avoid competing with crops like wheat, careful planning of planting and management is important. Their presence may also help reduce weeds and the need for herbicides before sowing (Bell, 2014; Ewing *et al.*, 2024; Millar & Badgery, 2009). Their perennial growth habit provides additional environmental benefits, including increased levels of soil carbon, improved soil stability (resistance to erosion), increased water retention, higher levels of biodiversity, and associated reduced input requirements, supporting sustainable land use (Bergquist *et al.*, 2025). These species may be particularly suited to low-productivity areas on farms where conventional crops are less viable, although their establishment and maintenance could present practical challenges.

The functional and nutritional profiles of the native grains suggest opportunities for targeted food applications. Weeping Grass, with high water and oil absorption and elevated β-glucan, may be suited for gluten-free breads, health-focused products, and as a source of viscosity and structure in cereal-based beverages. Curly Mitchell Grass, with high protein content and strong foaming capacity, could be developed into high-protein health or energy products, as well as aerated bakery items. Native Millet, with relatively high fat content and good hydration properties, could be used in flat breads and snack blends, while Button Grass, with moderate hydration and oil-binding traits, may be applied in flour blends or extruded snacks.

7.2 Future Directions

7.2.1 Environmental Evaluation and Agronomic Performance

Although this study characterised the biochemical and functional properties of the grains of four Australian native grass species, their response to environmental variation remains unexamined. To address this, future work should include both multi-location field trials and the sampling of grains from plants grown across different environments and seasons. This approach would enable a detailed evaluation of key agronomic traits, including yield, stress tolerance, pest resistance, nutrient efficiency, and grain quality.

These trials would enable identification of genotype × environment (G×E) interactions, which are important for evaluating consistent performance across regions. However, comparing different native grain species—or comparing them to domesticated cereals—poses specific challenges. Each species has distinct growth cycles and environmental requirements; for example, multi-environment trials in domesticated cereals, such as wheat, show that variation in sowing and maturity timing can drastically affect yield outcomes (Saeidnia *et al.*, 2023). This means that, unlike trials involving lines of a single species, multi-species field trials require designs that reflect the variation in growth cycles and harvesting schedules. Recognising these differences is crucial for meaningful trial design and data interpretation.

Native grasses could be strategically integrated into low-productivity zones on farms—areas often unsuitable for intensive cropping—due to their resilience and perennial growth habit. Morphological and physiological traits, along with water-use efficiency of native grains, offer insight into their adaptability and their suitability for low-input or climate-resilient farming systems. Integration of this agronomic information with biochemical and proteomic profiles can support the selection of superior genotypes and the development of breeding programs focused on improving yield stability and grain quality for commercial-scale cultivation.

7.2.2 Expanding Genomic Information

Genome sequencing will establish the foundation for trait discovery in native species by identifying genes linked to agronomic performance, stress tolerance, and nutritional value. It will facilitate pathway analysis and enable researchers to investigate molecular mechanisms that control physiological processes such as drought response, nutrient metabolism, and seed development. The availability of complete or high-quality reference genomes remarkably improves the accuracy of protein identification, annotation, and functional classification in proteomic studies. These resources also enhance other molecular approaches such as transcriptomics and metabolomics, and support precise gene discovery and biochemical pathway reconstruction.

These genomic tools—such as reference genomes and gene annotations—support targeted breeding strategies and advance both the potential development of improved native grain lines and the understanding of their unique evolutionary and adaptive traits by enhancing the resolution and reliability of molecular studies (Abbas *et al.*, 2024; Satrio *et al.*, 2024).

Wild grasses often exhibit greater genetic diversity than domesticated cereals due to the absence of intensive selection and breeding. This genetic diversity represents an important source of traits that can improve resilience and adaptability—for example, *Aegilops tauschii*, the diploid ancestral donor of the D genome in bread wheat, has been used to introduce disease resistance into modern wheat cultivars (Nevo, 1998; Woodhouse & Hufford, 2019; Zhao *et al.*, 2017).

7.2.3 Functional Property Optimisation

Future studies should comprehensively evaluate the behaviour of native grain flours or seeds within various food products, including bread, snacks, pasta, beverages, and fermented foods. Detailed rheological analyses are necessary to understand dough and batter properties, combined with product development trials aimed at optimising formulations and processing conditions.

Shelf-life evaluations determine product stability and safety over time, whereas sensory assessments provide valuable insights into consumer acceptance, preferences, and potential market success. These studies will establish the suitability of native grains for diverse food applications and guide their effective incorporation into commercial products, particularly by linking functional properties such as water absorption, foaming capacity, and viscosity to specific product requirements like dough stability, shelf-life, and texture in baked or fermented foods.

Addressing the challenges presented by various industrial applications will also require further investigation to optimise the functional properties of native grain flour. This process involves evaluating various processing methods, including sprouting, enzymatic treatment, and fermentation, to improve key functional properties. Sprouting, for instance, has been shown to increase enzymatic activity and improve nutrient availability in cereal grains (Mbithi-Mwikya *et al.*, 2000) and may be particularly useful for enhancing the digestibility of native grains with dense kernels and tightly packed endosperm, such as Native Millet (Chapter 2).

Enzymatic treatments can alter starch and protein structures, thereby modifying water and oil absorption capacity (Suri & Singh, 2023). Considering the relatively high amylose content and protein levels in some native grains (e.g., Curly Mitchell Grass), enzymatic modification targeting starch branching or partial protein hydrolysis could improve their hydration and foaming properties.

Fermentation can improve sensory characteristics and affect the rheological properties and foaming capacity of the flours. In flours with high beta-glucan content, such as Weeping Grass, dough viscosity is initially elevated due to strong water-binding capacity. This can reduce gas retention or slow fermentation. However, depending on fermentation conditions and microbial activity, partial degradation of beta-glucans may occur, as observed in oat sourdoughs fermented for 8 h, where viscosity remained high but beta-glucan molecular weight decreased slightly (Lu *et al.*, 2019). This moderate breakdown may help balance dough handling, nutritional benefits, and structural properties, particularly in gluten-free applications.

Applying these modifications allows native grain functionality to be adapted more effectively for the development of gluten-free or high-fibre food products, which are experiencing increased demand due to their health benefits and suitability for consumers with specific dietary needs. Targeted processing strategies may increase the industrial use of native grains, support their inclusion in a broader range of food products, and help maintain or improve product quality and consumer acceptance.

7.2.4 Ethical and Collaborative Development

The research and commercialisation of Australian native grains must be grounded in respect for the cultural, ecological, and historical knowledge of Indigenous communities. Recognition of Indigenous custodianship serves as both an ethical duty and a fundamental basis for socially responsible and sustainable science. Indigenous knowledge systems provide invaluable insights into sustainable land management, plant utilisation, and ecological resilience, substantially enriching scientific understanding and promoting innovation. During this PhD, consultation with a small number of Indigenous community members in Narrabri provided valuable perspectives that informed parts of the research.

Genuine, long-term partnerships with First Nations peoples and Torres Strait Islander communities are essential for guiding future research. Research should be designed collaboratively, with support given to Indigenous-led initiatives and fair benefit-sharing arrangements established. These partnerships ensure that the benefits arising from native grain development are equitably shared and that Indigenous voices remain central throughout the research and commercialisation processes.

A culturally respectful approach strengthens the legitimacy of research and improves its long-term impact on the development of native grains. It builds trust, facilitates knowledge exchange, and aids the preservation of Indigenous heritage, while simultaneously promoting economic opportunities within Indigenous communities. Incorporating Indigenous perspectives into native grain research ensures that scientific progress aligns with the principles of social justice and environmental sustainability.

References

Abah, C., Ishiwu, C., Obiegbuna, J., & Oladejo, A. (2020). Sorghum grains: nutritional composition, functional properties and its food applications. *European Journal of Nutrition & Food Safety*, *12*(5), 101-111.

ABARES. (2024). *Australian Crop Report September 2024*. ABARES.

ABARES. (2025). *Snapshot of Australian Agriculture 2025*. ABARES.

Abbas, Q., Wilhelm, M., Kuster, B., Poppenberger, B., & Frishman, D. (2024). Exploring crop genomes: assembly features, gene prediction accuracy, and implications for proteomics studies. *BMC Genomics*, *25*(1), 619.

Abd Elmoneim, O. E., & Bernhardt, R. (2010). Influence of grain germination on functional properties of sorghum flour. *Food Chemistry*, *121*(2), 387-392.

Abedi, F., Keitel, C., Khoddami, A., Marttila, S., Pattison, A. L., & Roberts, T. H. (2023). Indigenous Australian grass seeds as grains: macrostructure, microstructure and histochemistry. *AoB Plants*, *15*(6), plad071.

Adebo, J. A., & Kesa, H. (2023). Evaluation of nutritional and functional properties of anatomical parts of two sorghum (Sorghum bicolor) varieties. *Heliyon*, *9*(6).

Adiamo, O. Q., Phan, A. D. T., Akter, S., Sivakumar, D., Seke, F., Cunningham, P., & Sultanbawa, Y. (2024). Domesticated Australian wattle seeds (Acacia species): nutritional values, techno-functional properties and toxicological assessments after roasting. *International Journal of Food Science and Technology*, *59*(1), 573-583.

AgriFutures Australia. (2022). *Revitalised native grain industry gaining momentum*.

AgriFutures Australia. (2023). *Modernising Indigenous Native Grains Processing*.

Alpuche-Solís, A. G., & Paredes-López, O. (2000). Biotechnology to improve shelf life and quality traits of foods. In *Food Shelf Life Stability* (pp. 335-376). CRC Press.

Alvarez-Jubete, L., Arendt, E. K., & Gallagher, E. (2010). Nutritive value of pseudocereals and their increasing use as functional gluten-free ingredients. *Trends in Food Science & Technology*, *21*(2), 106-113.

Alves, T. O., D'Almeida, C. T., Scherf, K. A., & Ferreira, M. S. (2019). Modern approaches in the identification and quantification of immunogenic peptides in cereals by LC-MS/MS. *Frontiers in Plant Science*, *10*, 1470.

American Association of Cereal Chemists-Intrnational. (2000). Moisture – Air Oven Methods – AACC-I Method 44-15A.

Antonini, E., Zara, C., Valentini, L., Gobbi, P., Ninfali, P., & Menotta, M. (2018). Novel insights into pericarp, protein body globoids of aleurone layer, starchy granules of three cereals gained using atomic force microscopy and environmental scanning electronic microscopy. *European Journal of Histochemistry*, *62*(1), 20-27.

Arora, K., Tlais, A. Z. A., Augustin, G., Grano, D., Filannino, P., Gobbetti, M., & Di Cagno, R. (2023). Physicochemical, nutritional, and functional characterization of gluten-free ingredients and their impact on the bread texture. *LWT*, *177*, 114566.

Arp, C. G., Correa, M. J., & Ferrero, C. (2021). Resistant starches: A smart alternative for the development of functional bread and other starch-based foods. *Food Hydrocolloids*, *121*, 106949.

Asrarkulova, A., & Bulushova, N. (2018). Wheat gluten and its hydrolysates. Possible fields of practical use. *Applied Biochemistry and Microbiology*, *54*, 825-833.

Attard, B. (2008). *The economic history of Australia from 1788: An introduction*. Economic History Association. Retrieved June 2024 from

Australia's Virtual Herbarium. (2011). *Distribution map of Astrebla lappacea, Dactyloctenium radulans, and Microlaena stipoides*.

Australian Biological Resources Study. (n.d.). *Panicum decompositum species profile*.

Awuchi, C. G., Igwe, V. S., & Echeta, C. K. (2019). The functional properties of foods and flours. *International Journal of Advanced Academic Research*, *5*(11).

Babu, V., Rajan, V., & D'Silva, P. (2009). *Arabidopsis thaliana* J-class heat shock proteins: cellular stress sensors. *Functional & integrative genomics*, *9*, 433-446.

Bagheri, R., & Seyedein, S. M. (2011). The effect of adding rice bran fibre on wheat dough performance and bread quality. *World Applied Sciences Journal*, *14*(14), 121-125.

Baik, B.-K., & Ullrich, S. E. (2008). Barley for food: Characteristics, improvement, and renewed interest. *Journal of Cereal Science*, *48*(2), 233-242.

Bala, M., Handa, S., & Singh, R. (2020). Physicochemical, functional and rheological properties of grass pea (Lathyrus sativus L.) flour as influenced by particle size. *Heliyon*, *6*(11).

Barber, T. M., Kabisch, S., Pfeiffer, A. F., & Weickert, M. O. (2020). The health benefits of dietary fibre. *Nutrients*, *12*(10), 3209.

Barros, J. H., Telis, V. R., Taboga, S., & Franco, C. M. (2018). Resistant starch: effect on rheology, quality, and staling rate of white wheat bread. *Journal of Food Science and Technology*, *55*, 4578-4588.

Barthole, G., Lepiniec, L., Rogowsky, P. M., & Baud, S. (2012). Controlling lipid accumulation in cereal grains. *Plant Science*, *185*, 33-39.

Bell, L. (2014). *Economics and system applications for perennial grain crops in dryland farming systems in Australia*.

Bell, T. L., Pattison, A., Keitel, C., Khoddami, A., & Cross, R. (2022). Native grasses as a traditional and emerging source of food. *Australasian Plant Conservation: Journal of the Australian Network for Plant Conservation*, *31*(2), 3-6.

Bergquist, G., Sheaffer, C., Rakkar, M., Wyse, D., Jungers, J., & Gutknecht, J. (2025). Soil microbial and plant biomass carbon allocation within perennial and annual grain cropping systems. *Agriculture, Ecosystems & Environment*, *383*, 109535.

Bertoft, E. (2017). Understanding starch structure: Recent progress. *Agronomy*, *7*(3), 56.

Bhattarai, R. R., Dhital, S., Mense, A., Gidley, M. J., & Shi, Y.-C. (2018). Intact cellular structure in cereal endosperm limits starch digestion *in vitro*. *Food Hydrocolloids*, *81*, 139-148.

Biel, W., Jaroszewska, A., Stankowski, S., Sobolewska, M., & Kępińska-Pacelik, J. (2021). Comparison of yield, chemical composition and farinograph properties of common and ancient wheat grains. *European Food Research and Technology*, *247*(6), 1525-1538.

Binou, P., Yanni, A. E., Stergiou, A., Karavasilis, K., Konstantopoulos, P., Perrea, D., Tentolouris, N., & Karathanos, V. T. (2021). Enrichment of bread with beta-glucans or resistant starch induces similar glucose, insulin and appetite hormone responses in healthy adults. *European Journal of Nutrition*, *60*, 455-464.

Birch, J., Benkendorff, K., Liu, L., & Luke, H. (2023). The nutritional composition of Australian native grains used by First Nations people and their re-emergence for human health and sustainable food systems. *Frontiers in Sustainable Food Systems*, *7*, 1237862.

Bird, A. R., Vuaran, M., Brown, I., & Topping, D. L. (2007). Two high-amylose maize starches with different amounts of resistant starch vary in their effects on fermentation, tissue and digesta mass accretion, and bacterial populations in the large bowel of pigs. *British Journal of Nutrition*, *97*(1), 134-144.

Bliege Bird, R., Bird, D. W., Martine, C. T., McGuire, C., Greenwood, L., Taylor, D., Williams, T. M., & Veth, P. M. (2024). Seed dispersal by Martu peoples promotes the distribution of native plants in arid Australia. *Nature Communications*, *15*(1), 6019.

Brand-Miller, J. C., & Holt, S. H. (1998). Australian Aboriginal plant foods: a consideration of their nutritional composition and health implications. *Nutrition Research Reviews*, *11*(1), 5–23.

Brandolini, A., & Hidalgo, A. (2012). Wheat germ: not only a by-product. *International Journal of Food Sciences and Nutrition*, *63*(sup1), 71-74.

Burešová, I., Lullien-Pellerin, V., Červenka, L., Mlček, J., Šebestíková, R., & Masaříková, L. (2023). The comparison of the effect of flour particle size and content of damaged starch on rice and buckwheat slurry, dough, and bread characteristics. *Foods*, *12*(13), 2604.

Butcher, L. (2025). Growing the market for Indigenous grain foods: Product development and market segmentation in Australia. In *SAGE Business Cases*. SAGE Publications.

Buttriss, J., & Stokes, C. (2008). Dietary fibre and health: an overview. *Nutrition Bulletin*, *33*(3), 186-200.

Cane, S. (1987). Australian Aboriginal subsistence in the Western Desert. *Human Ecology*, *15*, 391-434.

Cappelli, A., Oliva, N., & Cini, E. (2020). A systematic review of gluten-free dough and bread: Dough rheology, bread characteristics, and improvement strategies. *Applied Sciences*, *10*(18), 6559.

Cavanagh, A. M., Godfree, R. C., & Morgan, J. W. (2019). An awn typology for Australian native grasses (*Poaceae*). *Australian Journal of Botany*, *67*(4), 309-334.

Chandra, S., Singh, S., & Kumari, D. (2015). Evaluation of functional properties of composite flours and sensorial attributes of composite flour biscuits. *Journal of Food Science and Technology*, *52*, 3681-3688.

Chapman, E. A., Thomsen, H. C., Tulloch, S., Correia, P. M., Luo, G., Najafi, J., DeHaan, L. R., Crews, T. E., Olsson, L., & Lundquist, P.-O. (2022). Perennials as future grain crops: opportunities and challenges. *Frontiers in Plant Science*, *13*, 898769.

Chen, G., Stump, M., & Li, Y. (2025). Protein Fractionation Using Osborne Sequential Extraction. In *Plant-Based Proteins* (pp. 69-74). Springer.

Chen, K., Yin, Y., Liu, S., Guo, Z., Zhang, K., Liang, Y., Zhang, L., Zhao, W., Chao, H., & Li, M. (2019). Genome-wide identification and functional analysis of oleosin genes in *Brassica napus* L. *BMC Plant Biology*, *19*, 1-20.

Chen, X., Lin, S., Liu, Q., Huang, J., Zhang, W., Lin, J., Wang, Y., Ke, Y., & He, H. (2014). Expression and interaction of small heat shock proteins (sHsps) in rice in response to heat stress. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1844*(4), 818-828.

Chen, Y., Mcpherson, A., Radosavljevic, M., Lee, V., Wong, K., & Jane, J. (1998). Effects of starch chemical structures on gelatinization and pasting properties. *Żywność Technol Jakość*, *17*, 63-71.

Chen, Y., Wang, J., Yao, L., Li, B., Ma, X., Si, E., Yang, K., Li, C., Shang, X., & Meng, Y. (2022). Combined proteomic and metabolomic analysis of the molecular mechanism underlying the response to salt stress during seed germination in barley. *International Journal of Molecular Sciences*, *23*(18), 10515.

Chivers, I., Warrick, R., Bornman, J., & Evans, C. (2015). Native grasses make new products. In: RIRDC, Canberra, June.

Ciecierska, A., Drywien, M., Hamulka, J., & Sadkowski, T. (2019). Nutraceutical functions of beta-glucans in human nutrition. *Roczniki Państwowego Zakładu Higieny*, *70*(4).

Clarkson, C., Jacobs, Z., Marwick, B., Fullagar, R., Wallis, L., Smith, M., Roberts, R. G., Hayes, E., Lowe, K., & Carah, X. (2017). Human occupation of northern Australia by 65,000 years ago. *Nature*, *547*(7663), 306-310.

Coeliac Australia. (n.d.). *Making a Gluten Free Claim*. https://coeliac.org.au/for-business/making-a-gluten-free-claim/

Cole, I., & Johnston, W. (2006). Seed production of Australian native grass cultivars: an overview of current information and future research needs. *Australian Journal of Experimental Agriculture*, *46*(3), 361-373.

Colgrave, M. L., Byrne, K., & Howitt, C. A. (2017). Food for thought: Selecting the right enzyme for the digestion of gluten. *Food Chemistry*, *234*, 389-397.

Combe, A. L., Ang, J. K., & Bamforth, C. W. (2013). Positive and negative impacts of specialty malts on beer foam: A comparison of various cereal products for their foaming properties. *Journal of the Science of Food and Agriculture*, *93*(9), 2094-2101.

Courtin, C., & Delcour, J. A. (2002). Arabinoxylans and endoxylanases in wheat flour bread-making. *Journal of Cereal Science*, *35*(3), 225-243.

Cowley, J. M., Phillips, A. L., Khor, S. F., Neumann, T., Lim, W. L., & Burton, R. A. (2023). Carbohydrates in Kangaroo grass (*Themeda triandra* Forssk.) grain and perspectives on its food potential. *Journal of Cereal Science*, *111*, 103670.

Cuevas, R. P., Pede, V. O., McKinley, J., Velarde, O., & Demont, M. (2016). Rice grain quality and consumer preferences: a case study of two rural towns in the Philippines. *PLoS One*, *11*(3), e0150345.

Curran, G., Barwick, L., Turpin, M., Walsh, F., & Laughren, M. (2019). Central Australian Aboriginal songs and biocultural knowledge: evidence from women's ceremonies relating to edible seeds. *Journal of Ethnobiology*, *39*(3), 354-370.

DFAT. (2001). *Australia's Trades Since Federation*. Commonwealth of Australia.

Dhingra, D., Michael, M., Rajput, H., & Patil, R. (2012). Dietary fibre in foods: a review. *Journal of Food Science and Technology*, *49*(3), 255-266.

Din, A., Chughtai, M., Khan, M., Shahzad, A., Khaliq, A., & Nasir, M. (2018). Nutritional and functional perspectives of barley β-glucan. *International Food Research Journal*, *25*(5), 1773-1784.

Ding, F., Li, F., & Zhang, B. (2022). A plastid-targeted heat shock cognate 70-kDa protein confers osmotic stress tolerance by enhancing ROS scavenging capability. *Frontiers in Plant Science*, *13*, 1012145.

Drake, A., Keitel, C., & Pattison, A. (2021). The use of Australian native grains as a food: a review of research in a global grains context. *The Rangeland Journal*, *43*(4), 223–233.

Dyson, L. E. (2006). Indigenous Australian cookery, past and present. *Journal of Australian Studies*, *30*(87), 5-18.

Edwards, M. A., Osborne, B. G., & Henry, R. J. (2008). Effect of endosperm starch granule size distribution on milling yield in hard wheat. *Journal of Cereal Science*, *48*(1), 180-192.

Ee, K. Y., Eng, M. K., & Lee, M. L. (2020). Physicochemical, thermal and rheological properties of commercial wheat flours and corresponding starches. *Food Science and Technology*, *40*, 51-59.

EFSA Panel on Dietetic Products, N., & Allergies. (2010). Scientific Opinion on the substantiation of a health claim related to oat beta glucan and lowering blood cholesterol and reduced risk of (coronary) heart disease pursuant to Article 14 of Regulation (EC) No 1924/2006. *EFSA Journal*, *8*(12), 1885.

El Khoury, D., Cuda, C., Luhovyy, B., & Anderson, G. (2012). Beta-glucan: health benefits in obesity and metabolic syndrome. *Journal of Nutrition and Metabolism*, *2012*(1), 851362.

Elleuch, M., Bedigian, D., Roiseux, O., Besbes, S., Blecker, C., & Attia, H. (2011). Dietary fibre and fibre-rich by-products of food processing: Characterisation, technological functionality and commercial applications: A review. *Food Chemistry*, *124*(2), 411-421.

Ellis, H., Rosen-Bronson, S., O'Reilly, N., & Ciclitira, P. (1998). Measurement of gluten using a monoclonal antibody to a coeliac toxic peptide of A gliadin. *Gut*, *43*(2), 190-195.

Endalamaw, C., Tsegaye, D., van Biljon, A., Herselman, L., & Labuschagne, M. (2025). Kernel composition in sorghum landraces revealed via analyses of genotype-by-environment interactions. *PloS One*, *20*(4), e0320513.

Ens, E., WAlsh, F., & Clarke, P. (2017). Aboriginal people and Australia's vegetation: past and current interactions. *Australian vegetation*, 89-112.

Eraniappan, S., Balasubramaniyan, P., Uddandrao, V. S., Roy, A., & Singaravel, S. (2023). Beta-glucan suppresses high-fat-diet-induced obesity by attenuating dyslipidemia and modulating obesogenic marker expressions in rats. *Fundamental & Clinical Pharmacology*, *37*(3), 629-638.

Evans, D. E., & Bamforth, C. W. (2009). Beer foam: Achieving a suitable head. In *Beer: A quality perspective* (pp. 1-60). Academic Press.

Evers, A., O'brien, L., & Blakeney, A. (1999). Cereal structure and composition. *Australian Journal of Agricultural Research*, *50*(5), 629-650.

Evers, T., & Millar, S. (2002). Cereal grain structure and development: some implications for quality. *Journal of Cereal Science*, *36*(3), 261-284.

Ewing, P. M., Chim, B. K., Lehman, R. M., & Osborne, S. L. (2024). Diversified grain rotations can be highly and reliably productive in unstable climates. *Field Crops Research*, *310*, 109361.

Farooq, M., Rehman, A., Li, X., & Siddique, K. H. (2023). Neglected and underutilized crops and global food security. In *Neglected and Underutilized Crops* (pp. 3-19). Elsevier.

Fincher, G., & Stone, B. (1986). Cell walls and their components in cereal grain technology. *Advances in Cereal Science and Technology*, *8*, 207-295.

Fletcher, M.-S., Hall, T., & Alexandra, A. N. (2021). The loss of an indigenous constructed landscape following British invasion of Australia: An insight into the deep human imprint on the Australian landscape. *Ambio*, *50*(1), 138-149.

Flores-Jiménez, N. T., Ulloa, J. A., & Urías-Silvas, J. E. (2024). Assessment of the physicochemical, functional and structural characteristics of a defatted flour from guamuchil (*Pithecellobium dulce* (Roxb.) seeds. *Future Foods*, *9*, 100351.

Flores-Pérez, Ú., & Jarvis, P. (2013). Molecular chaperone involvement in chloroplast protein import. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1833*(2), 332-340.

Foster, P. R., Reseigh, J., & Myers, R. J. (2010). *An Introduction to the Nutritional Composition of Australian Native Grasses: Forage and Seed* (First ed.). Rural Solutions SA.

Friday, O., Monday, A., & Jessica, O. (2023). Effects of Bran and Hull Retention on the Sensory and Nutrients Composition of "Ogi Flour" Prepared from Maize, Millet and Sorghum. *Asian Food Science Journal*, *22*(1), 9-18.

Fuentes-Zaragoza, E., Riquelme-Navarrete, M., Sánchez-Zapata, E., & Pérez-Álvarez, J. (2010). Resistant starch as functional ingredient: A review. *Food Research International*, *43*(4), 931-942.

Furlán, L. T. R., Padilla, A. P., & Campderrós, M. E. (2015). Improvement of gluten-free bread properties by the incorporation of bovine plasma proteins and different saccharides into the matrix. *Food Chemistry*, *170*, 257-264.

Ganesan, V., Subbaiah, B., Stephen, N. M., Dhanushkodi, M., Kolandaivadivel, R., & Veeramani, R. (2021). Optimization of extrusion process using response surface methodology for producing squid and millet based extrudate. *Turkish Journal of Fisheries and Aquatic Sciences*, *22*(2).

Garutti, M., Nevola, G., Mazzeo, R., Cucciniello, L., Totaro, F., Bertuzzi, C. A., Caccialanza, R., Pedrazzoli, P., & Puglisi, F. (2022). The impact of cereal grain composition on the health and disease outcomes. *Frontiers in Nutrition*, *9*, 888974.

Gasparre, N., Rosell, C. M., & Boukid, F. (2024). The growing popularity of low-carb cereal-based products: the lay of the land. *European Food Research and Technology*, *250*(2), 455–467.

Gibson, T., McCleary, B., & Solah, V. (1995). Con A revisited a simplified assay to measure amylose in cereal starches. Proc. 45th Australian Cereal Chemistry Conference,

Gidley, M. J., & Yakubov, G. E. (2019). Functional categorisation of dietary fibre in foods: Beyond 'soluble'vs 'insoluble'. *Trends in Food Science & Technology*, *86*, 563-568.

Gill, S. K., Rossi, M., Bajka, B., & Whelan, K. (2021). Dietary fibre in gastrointestinal health and disease. *Nature Reviews Gastroenterology & Hepatology*, *18*(2), 101-116.

Gómez, M., Ronda, F., Blanco, C. A., Caballero, P. A., & Apesteguía, A. (2003). Effect of dietary fibre on dough rheology and bread quality. *European Food Research and Technology*, *216*(1), 51-56.

Gorman, J., Pearson, D., & Wurm, P. (2020). Old Ways, New Ways—Scaling Up from Customary Use of Plant Products to Commercial Harvest Taking a Multifunctional, Landscape Approach. *Land*, *9*(5), 171.

Gott, B. (2008). Indigenous use of plants in south-eastern Australia. *Telopea*, *12*(2), 215-226.

Gott, B., Williams, N. S., & Antos, M. (2015). Humans and grasslands-a social history. *Land of sweeping plains: Managing and restoring the native Grasslands of south-eastern Australia*, 9-10.

Govindaraj, M., Pattanashetti, S. K., Patne, N., Kanatti, A. A., & Ciftci, Y. (2018). Breeding cultivars for heat stress tolerance in staple food crops. *Next Generation Plant Breeding. London: IntechOpen*, 45-74.

Grain & Legumes Nutrition Council. (2010). *Grain & Legumes Health Report*.

Grains & Legumes Nutrition Council. (2023a). *About whole grains*.

Grains & Legumes Nutrition Council. (2023b). *Whole Grains for a Healthier Future*.

Guleria, P., Kumari, S., & Dangi, N. (2015). β-glucan: health benefits and role in food industry-a review. *International Journal of Enhanced Research in Science Technology & Engineering*, *4*(8), 3-7.

Hajkowicz, S., & Young, M. (2005). Costing yield loss from acidity, sodicity and dryland salinity to Australian agriculture. *Land Degradation & Development*, *16*(5), 417-433.

Hallström, E., Sestili, F., Lafiandra, D., Björck, I., & Östman, E. (2011). A novel wheat variety with elevated content of amylose increases resistant starch formation and may beneficially influence glycaemia in healthy subjects. *Food & Nutrition Research*, *55*(1), 7074.

Hayes, E. H., Fullagar, R., Field, J. H., Coster, A. C., Matheson, C., Nango, M., Djandjomerr, D., Marwick, B., Wallis, L. A., & Smith, M. A. (2022). 65,000-years of continuous grinding stone use at Madjedbebe, Northern Australia. *Scientific Reports*, *12*(1), 11747.

Heiniö, R.-L., Noort, M., Katina, K., Alam, S. A., Sozer, N., De Kock, H. L., Hersleth, M., & Poutanen, K. (2016). Sensory characteristics of wholegrain and bran-rich cereal foods–a review. *Trends in Food Science & Technology*, *47*, 25-38.

Henzell, T. (2007). *Australian agriculture: its history and challenges*. CSIRO publishing.

Herburger, K., & Holzinger, A. (2016). Aniline blue and Calcofluor white staining of callose and cellulose in the streptophyte green algae *Zygnema* and *Klebsormidium*. *Bio-protocol*, *6*(20), e1969-e1969.

Hojsak, I., Benninga, M. A., Hauser, B., Kansu, A., Kelly, V. B., Stephen, A. M., Lopez, A. M., Slavin, J., & Tuohy, K. (2022). Benefits of dietary fibre for children in health and disease. *British Medical Journal*, *107*(11), 973-979.

Holtekjølen, A., Olsen, H., Færgestad, E., Uhlen, A., & Knutsen, S. (2008). Variations in water absorption capacity and baking performance of barley varieties with different polysaccharide content and composition. *LWT-Food Science and Technology*, *41*(10), 2085-2091.

Horton, D. (1982). The burning question: Aborigines, fire and Australian ecosystems. *Mankind*, *13*(3), 237-252.

Hughes, L., Steffen, W., Rice, M., & Pearce, A. (2015). *Feeding a hungry nation: Climate change, food and farming in Australia*. Climate Council of Australia.

Irving, D., & Jideani, I. (1997). Microstructure and composition of *Digitaria exilis* Stapf (acha): a potential crop. *Cereal Chemistry*, *74*(3), 224-228.

Izydorczyk, M., & Biliaderis, C. (2007). Arabinoxylans: Technologically and nutritionally functional plant polysaccharides. *Functional Food Carbohydrates*, 249-290.

Jääskeläinen, A.-S., Holopainen-Mantila, U., Tamminen, T., & Vuorinen, T. (2013). Endosperm and aleurone cell structure in barley and wheat as studied by optical and Raman microscopy. *Journal of Cereal Science*, *57*(3), 543-550.

Jacobs, S. W., Whalley, R., & Wheeler, D. J. (2008). *Grasses of New South Wales* (4th ed.). University of New England

Jain, D., Singh, P., Rani, K., & Prajapati, A. (2025). Oat, a Distinct Cereal: Origin, History, Production Practices, and Production Economics. In *Oat (Avena sativa)* (pp. 1-46). CRC Press.

Janssen, F., Breugelmans, A., Gunes, D. Z., & Wouters, A. G. (2025). Relating the foaming properties of oat proteins to their air-water interfacial characteristics and thin film drainage dynamics. *Food Hydrocolloids*, 111426.

Jenifer, J., Bell, T. L., Khoddami, A., & Pattison, A. L. (2023). *Panicum decompositum*, an Australian Native Grass, Has Strong Potential as a Novel Grain in the Modern Food Market. *Foods*, *12*(10), 2048.

Jenkins, T., Landschoot, S., Dewitte, K., Haesaert, G., Reade, J., & Randall, N. (2023). Evidence of development of underutilised crops and their ecosystem services in Europe: a systematic mapping approach. *CABI Agriculture and Bioscience*, *4*(1), 55.

Joye, I. J. (2020). Dietary fibre from whole grains and their benefits on metabolic health. *Nutrients*, *12*(10), 3045.

Kamal-Eldin, A., Lærke, H. N., Knudsen, K.-E. B., Lampi, A.-M., Piironen, V., Adlercreutz, H., Katina, K., Poutanen, K., & Åman, P. (2009). Physical, microscopic and chemical characterisation of industrial rye and wheat brans from the Nordic countries. *Food & Nutrition Research*, *53*(1), 1912.

Kasem, S., Waters, D., Rice, N., Shapter, F. M., & Henry, R. J. (2011a). The endosperm morphology of rice and its wild relatives as observed by scanning electron microscopy. *Rice*, *4*(1), 12-20.

Kasem, S., Waters, D., Rice, N., Shapter, F. M., & Henry, R. J. (2011b). The endosperm morphology of rice and its wild relatives as observed by scanning electron microscopy. *Rice*, *4*, 12-20.

Kaur, R., Sharma, M., Ji, D., Xu, M., & Agyei, D. (2019). Structural features, modification, and functionalities of beta-glucan. *Fibers*, *8*(1), 1.

Kaur, S., Kaur, G., Kumari, A., Ghosh, A., Singh, G., Bhardwaj, R., Kumar, A., & Riar, A. (2025). Resurrecting Forgotten Crops: Food-Based Products from Potential Underutilized Crops–A Path to Nutritional Security and Diversity. *Future Foods*, 100585.

Kaushik, R., Sharma, N., Swami, N., Sihag, M., Goyal, A., Chawla, P., Kumar, A., & Pawar, A. (2013). Physico-chemical properties, extraction and characterization of gluten from different Indian wheat cultivars. *Research & Reviews: A Journal of Crop Science and Technology*, *2*(1), 1-6.

Khalid, A., Hameed, A., & Tahir, M. F. (2023). Wheat quality: A review on chemical composition, nutritional attributes, grain anatomy, types, classification, and function of seed storage proteins in bread making quality. *Frontiers in Nutrition*, *10*, 1053196.

Khan, Z., & Shahwar, D. (2020). Role of heat shock proteins (HSPs) and heat stress tolerance in crop plants. *Sustainable Agriculture in the Era of Climate Change*, 211-234.

Kim, Y., & Flores, R. (1999). Determination of bran contamination in wheat flours using ash content, color, and bran speck counts. *Cereal Chemistry*, *76*(6), 957-961.

Kimber, R. (1983). Black lightning: Aborigines and fire in central Australia and the Western Desert. *Archaeology in Oceania*, *18*(1), 38-45.

Kinsella, J. E., & Melachouris, N. (1976). Functional properties of proteins in foods: a survey. *Critical Reviews in Food Science & Nutrition*, *7*(3), 219-280.

Koehler, P., & Wieser, H. (2013). Chemistry of cereal grains. In M. Gobbetti & M. Ganzle (Eds.), *Handbook on Sourdough Biotechnology* (pp. 11–45). Springer.

Kondo, T., Crisp, M. D., Linde, C., Bowman, D. M., Kawamura, K., Kaneko, S., & Isagi, Y. (2012). Not an ancient relic: the endemic Livistona palms of arid central Australia could have been introduced by humans. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1738), 2652-2661.

Konik, C. M., Miskelly, D. M., & Gras, P. W. (1993). Starch swelling power, grain hardness and protein: Relationship to sensory properties of Japanese noodles. *Starch-Stärke*, *45*(4), 139-144.

Korres, N. E., Norsworthy, J. K., Tehranchian, P., Gitsopoulos, T. K., Loka, D. A., Oosterhuis, D. M., Gealy, D. R., Moss, S. R., Burgos, N. R., & Miller, M. R. (2016). Cultivars to face climate change effects on crops and weeds: a review. *Agronomy for Sustainable Development*, *36*(1), 12.

Labuschagne, M. (2018). A review of cereal grain proteomics and its potential for sorghum improvement. *Journal of Cereal Science*, *84*, 151-158.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680-685.

Lafond, G., & Baker, R. (1986). Effects of genotype and seed size on speed of emergence and seedling vigor in nine spring wheat cultivars 1. *Crop Science*, *26*(2), 341-346.

Lamp, C. A., Forbes, S. J., & Cade, J. W. (1990). *Grasses of Temperate Australia. A Field Guide*. Inkata Press.

Lasztity, R. (2017). *The Chemistry of Cereal Proteins*. Routledge.

Latijnhouwers, M., Xu, X.-M., & Møller, S. G. (2010). Arabidopsis stromal 70-kDa heat shock proteins are essential for chloroplast development. *Planta*, *232*, 567-578.

Latz, P. (2007). *The Flaming Desert, Arid Australia—A Fire Shaped Landscape*. Peter Latz: Alice Springs, NT.

Latz, P. K. (1995). *Bushfires & Bushtucker: Aboriginal Plant Use in Central Australia*. IAD Press.

Lazaridou, A., & Biliaderis, C. (2007). Molecular aspects of cereal β-glucan functionality: Physical properties, technological applications and physiological effects. *Journal of Cereal Science*, *46*(2), 101-118.

Lebert, L., Buche, F., Sorin, A., & Aussenac, T. (2022). The wheat aleurone layer: optimisation of its benefits and application to bakery products. *Foods*, *11*(22), 3552.

Li, J., Liu, C., Yin, Y., Sun, X., & Wu, J. (2023). Characterization of ash content in wheat flour using data fusion. *Infrared Physics & Technology*, *133*, 104792.

Li, J., Yoshimura, K., Sasaki, M., & Maruyama, K. (2024). The Consumption of High-Amylose Rice and its Effect on Postprandial Blood Glucose Levels: A Literature Review. *Nutrients*, *16*(23), 4013.

Li, L., Liu, Z., Li, X., Chu, X., Yang, W., Wang, B., Xie, Y., & Li, X. (2023). Superior gluten structure and more small starch granules synergistically confer dough quality for high amylose wheat varieties. *Frontiers in Nutrition*, *10*, 1195505.

Liu, H., Zhu, J., Gao, R., Ding, L., Yang, Y., Zhao, W., Cui, X., Lu, W., Wang, J., & Li, Y. (2024). Estimating effects of whole grain consumption on type 2 diabetes, colorectal cancer and cardiovascular disease: a burden of proof study. *Nutrition Journal*, *23*(1), 49.

Liu, Q., Qiu, Y., & Beta, T. (2010). Comparison of antioxidant activities of different colored wheat grains and analysis of phenolic compounds. *Journal of Agricultural and Food Chemistry*, *58*(16), 9235-9241.

Liukkonen, K., & Laakso, S. (1992). Characterization of internal and surface lipids of oat starches from two isolation processes. *Starch-Stärke*, *44*(4), 128-132.

Local Land Services, N. G. (2015). Common native grasses of central west NSW.

Lopes, C. V. A., Mihrshahi, S., Ronto, R., & Hunter, J. (2023). Aboriginal food practices and Australian native plant-based foods: A step toward sustainable food systems. *Sustainability*, *15*(15), 11569.

Lovegrove, A., Wood, A. J., Hassall, K. L., Howes, L., Poole, M., Tosi, P., & Shewry, P. (2020). The contribution of fiber components to water absorption of wheat grown in the UK. *Cereal Chemistry*, *97*(5), 940-948.

Lu, J., Shan, L., Xie, Y., Min, F., Gao, J., Guo, L., Ren, C., Yuan, J., Gilissen, L., & Chen, H. (2019). Effect of fermentation on content, molecule weight distribution and viscosity of β-glucans in oat sourdough. *International Journal of Food Science and Technology*, *54*(1), 62-67.

Ma, S., Wang, C., Li, L., & Wang, X. (2020). Effects of particle size on the quality attributes of wheat flour made by the milling process. *Cereal Chemistry*, *97*(2), 172-182.

Mbithi-Mwikya, S., Van Camp, J., Yiru, Y., & Huyghebaert, A. (2000). Nutrient and antinutrient changes in finger millet (Eleusine coracan) during sprouting. *LWT-Food Science and Technology*, *33*(1), 9-14.

McCleary, B. V., & Codd, R. (1991). Measurement of (1→ 3),(1→ 4)-β-D-glucan in barley and oats: A streamlined enzymic procedure. *Journal of the Science of Food and Agriculture*, *55*(2), 303-312.

Mccleary, B. V., Gibson, T. S., & Mugford, D. C. (1997). Measurement of total starch in cereal products by amyloglucosidase-α-amylase method: Collaborative study. *Journal of AOAC International*, *80*(3), 571-579.

McCleary, B. V., McNally, M., & Rossiter, P. (2002). Measurement of resistant starch by enzymatic digestion in starch and selected plant materials: collaborative study. *Journal of AOAC International*, *85*(5), 1103-1111.

McLennon, E., Dari, B., Jha, G., Sihi, D., & Kankarla, V. (2021). Regenerative agriculture and integrative permaculture for sustainable and technology driven global food production and security. *Agronomy Journal*, *113*(6), 4541-4559.

Mildwaters, J., & Clarkson, C. (2020). An experimental assessment of the grinding characteristics of some native seeds used by Aboriginal Australians. *Journal of Archaeological Science: Reports*, *30*, 102127.

Millar, G., & Badgery, W. (2009). Pasture cropping: a new approach to integrate crop and livestock farming systems. *Animal Production Science*, *49*(10), 777-787.

Miller, J. B., James, K. W., & Maggiore, P. M. A. (1993). *Tables of Composition of Australian Aboriginal Foods*. Aboriginal Studies Press.

Min, D. B., & Ellefson, W. C. (2010). Fat Analysis. In S. S. Nielsen (Ed.), *Food Analysis* (pp. 117-132). Springer.

Moazzami, A. A., Lampi, A.-M., & Kamal-Eldin, A. (2011). Bioactive lipids in cereals and cereal products. *Fruit and Cereal Bioactives–Sources, Chemistry, and Applications*, 229-249.

Modipuram, M. (2013). Assessment of functional properties of different flours. *African Journal of Agricultural*, *38*(8), 4849-4852.

Mohebbi, Z., Homayouni, A., Azizi, M. H., & Hosseini, S. J. (2018). Effects of beta-glucan and resistant starch on wheat dough and prebiotic bread properties. *Journal of Food Science and Technology*, *55*, 101-110.

Mudgil, D. (2017). The interaction between insoluble and soluble fiber. In *Dietary fiber for the prevention of cardiovascular disease* (pp. 35-59). Elsevier.

Musigakun, P., & Thongngam, M. (2007). Characteristics and functional properties of sorghum protein (kafirin). *Agriculture and Natural Resources*, *41*(5), 313-318.

Nakashima, K., & Yamaguchi-Shinozaki, K. (2013). ABA signaling in stress-response and seed development. *Plant Cell Reports*, *32*, 959-970.

Nandane, A. S., Ganorkar, P. M., Ranveer, R. C., Patil, H., Al-Asmari, F., Sangsawad, P., Nirmal, N., & Ozogul, F. (2025). Impact of Extrusion Process on the Macro-and Micro-nutrient in Extruded Food Products: Challenges and Future Trends. *Food and Bioprocess Technology*, 1-25.

National Health and Medical Research Council. (2013). *Australian Dietary Guidlines*.

Neave, M., & Rayburg, S. (2006). Salinity and erosion: a preliminary investigation of soil erosion on a salinized hillslope. *IAHS Publication*, *306*, 531.

Nevo, E. (1998). Genetic diversity in wild cereals: regional and local studies and their bearing on conservation ex situ and in situ. *Genetic Resources and Crop Evolution*, *45*, 355-370.

Newell, M. T. (2021). *Proceedings of the Perennial Artisan Grains Workshop*. AgriFutures Australia.

Niazi, M. K., Saeed, Z., Imran, S., & Hassan, F. (2023). Therapeutic Effect of Barley on Cardiovascular Diseases. In L. Xianli (Ed.), *Frontiers in Clinical Trials*. IntechOpen.

Nicholas, R., Williams, D. W., & Hunter, P. (1994). Investigation of the value of β-glucan-specific fluorochromes for predicting the β-glucan content of the cell walls of zoopathogenic fungi. *Mycological Research*, *98*(6), 694-698.

Nieto-Nieto, T. V., Wang, Y. X., Ozimek, L., & Chen, L. (2014). Effects of partial hydrolysis on structure and gelling properties of oat globular proteins. *Food Research International*, *55*, 418-425.

Nissar, J., Ahad, T., Naik, H., & Hussain, S. (2017). Resistant starch-chemistry and nutritional properties. *International Journal of Food Science and Nutrition*, *2*(6), 95-108.

NSW Department of Primary Industries. (2024). *Wheat*.

O'Hara, I., Kent, G., Alberston, P., Harrison, M., Hobson, P., McKenzie, N., Moghaddam, L., Moller, D., Rainey, T., Stolz, W., Wong, H.-H., & Ellett, B. (2013). *Sweet sorghum – Opportunities for a new renewable fuel and food industry in Australia*. A. Australia.

Obadi, M., Qi, Y., & Xu, B. (2023). High-amylose maize starch: Structure, properties, modifications and industrial applications. *Carbohydrate Polymers*, *299*, 120185.

Olanca, B., & Özay, D. S. (2010). Preparation and functional properties of gluten hydrolysates with wheat-bug (Eurygaster spp.) Protease. *Cereal Chemistry*, *87*(6), 518-523.

Olsen, J. V., Ong, S.-E., & Mann, M. (2004). Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Molecular & Cellular Proteomics*, *3*(6), 608-614.

Osborne, T. (1907). *The Protein of the Wheat Kernel Publication* (First ed.). Carnegie Institution of Washington.

Osorio, C. E., Mejías, J. H., & Rustgi, S. (2019). Gluten detection methods and their critical role in assuring safe diets for celiac patients. *Nutrients*, *11*(12), 2920.

Ostapenko, D. (2013). Golden Horizons: Expansion of the Wheat-growing Industry in the Colony of Victoria in the 1850s. *Agricultural History*, *87*(1), 35-56.

Pascoe, B. (2024). Indigenous Agriculture and Sustainability Knowledge: The Great Australian Silence. In *Innovation Pathways to Sustainability* (pp. 208-217). Routledge.

Pattison, A., Keitel, C., Binge, H., Saunders, K., Hall, D. Y., Thistlethwaite, R., Onus, N., Badaoui, C., Booby, S., Foods, B. D., Ashby, R., & Birch, J. (2023). *Indigenous Grasslands for Grain*.

Pattison, A., Khoddami, A., & Keitel, C. (2020). *Native Grains from Paddock to Plate: Study of the Economic, Environmental and Social Sustainability of an Ancient System in a Modern Context*. S. I. o. Agriculture.

Pattison, A., McGee, K., Birch, J., Saunders, K., Ashby, R., Quinnell, R., Bell-Anderson, K., & Way, A. (2023). What Do We Know About Threshing Traditional Grains in Australia? *Journal of Ethnobiology*, *43*(4), 339–350.

Pattison, A., Thistlethwaite, R., Saunders, K., Onus, N., Hall, D., & Binge, H. (2025). *Modernising Indigenous Native Grains Processing*.

Perry, J., & Ying, W. (2016). A review of physiological effects of soluble and insoluble dietary fibers.

Pesonen, L., Romanelli, T., Colaço, A. F., Veiga, J. P., Sousa, A., da Silva, J. M., Serrano, J., Shahidian, S., da Silveira, D. L., & Simões, M. (2024). Smart farms: Improving data-driven decision making in agriculture.

Please, P. (2023). The feeling of salt, land, and water. In *Cultural Complexes in Australia* (pp. 75-95). Routledge.

Poutanen, K. S., Kårlund, A. O., Gómez-Gallego, C., Johansson, D. P., Scheers, N. M., Marklinder, I. M., Eriksen, A. K., Silventoinen, P. C., Nordlund, E., & Sozer, N. (2022). Grains–a major source of sustainable protein for health. *Nutrition Reviews*, *80*(6), 1648-1663.

Productivity Commission. (2005). *Trends in Australian Agriculture*. Commonwealth of Australia.

Prosky, L., Asp, N.-G., Schweizer, T. F., Devries, J. W., & Furda, I. (1988). Determination of insoluble, soluble, and total dietary fiber in foods and food products: interlaboratory study. *Journal of the Association of Official Analytical Chemists*, *71*(5), 1017-1023.

Qiu, X., Gong, R., Tan, Y., & Yu, S. (2012). Mapping and characterization of the major quantitative trait locus qSS7 associated with increased length and decreased width of rice seeds. *Theoretical and Applied Genetics*, *125*, 1717-1726.

Qureshi, M. E., Hanjra, M. A., & Ward, J. (2013). Impact of water scarcity in Australia on global food security in an era of climate change. *Food Policy*, *38*, 136-145.

Rahman, S., Copeland, L., Atwell, B. J., & Roberts, T. H. (2021). Elevated CO2 differentially affects the properties of grain from wild and domesticated rice. *Journal of Cereal Science*, *100*, 103227.

Rani, M., Singh, G., Siddiqi, R. A., Gill, B. S., Sogi, D. S., & Bhat, M. A. (2021). Comparative quality evaluation of physicochemical, technological, and protein profiling of wheat, rye, and barley cereals. *Frontiers in Nutrition*, *8*, 694679.

Rauch, J. N., Zuiderweg, E. R., & Gestwicki, J. E. (2016). Non-canonical interactions between heat shock cognate protein 70 (Hsc70) and Bcl2-associated anthanogene (BAG) co-chaperones are important for client release. *Journal of Biological Chemistry*, *291*(38), 19848-19857.

Redden, R., Norton, S., Whitehouse, K., & Slater, A. (2020). History of crop introductions, breeding and selection in Australia: The 140 years from 1788. *Agricultural Science*, *31*(2), 92-99.

Řehulková, H., Marchetti-Deschmann, M., Pittenauer, E., Allmaier, G., & Řehulka, P. (2009). Improved identification of hordeins by cysteine alkylation with 2-bromoethylamine, SDS-PAGE and subsequent in-gel tryptic digestion. *Journal of Mass Spectrometry*, *44*(11), 1613-1621.

Renuse, S., Harsha, H., Kumar, P., Acharya, P. K., Sharma, J., Goel, R., Kumar, G. S. S., Raju, R., Prasad, T. K., & Slotta, T. (2012). Proteomic analysis of an unsequenced plant—*Mangifera indica*. *Journal of Proteomics*, *75*(18), 5793-5796.

Respiratory Care WA. (2024). *Low allergen plants: A guide to asthma-friendly gardening*.

Reynolds, A., Mann, J., Cummings, J., Winter, N., Mete, E., & Te Morenga, L. (2019). Carbohydrate quality and human health: a series of systematic reviews and meta-analyses. *The Lancet*, *393*(10170), 434-445.

Rosa-Sibakov, N., Poutanen, K., & Micard, V. (2015). How does wheat grain, bran and aleurone structure impact their nutritional and technological properties? *Trends in Food Science & Technology*, *41*(2), 118-134.

Ryley, M. J., & Park, R. F. (2023). Stem rust of wheat in colonial Australia and the development of the plant pathology profession. *Historical Records of Australian Science*, *35*(2), 83-97.

Sablani, S. S., & Ramaswamy, H. S. (2003). Physical and thermal properties of cereal grains. *Hanbook of Postharvest Technology, eds GSV Raghavan, A. Chakraverty, AS Mujumdar and HS Ramaswamy*, 17-40.

Sadaka, S., & Rosentrater, K. A. (2022). The significance of moisture and its measurement in cereal grains and grain products. In *Storage of Cereal Grains and Their Products* (pp. 261-292). Elsevier.

Saeidnia, F., Taherian, M., & Nazeri, S. M. (2023). Graphical analysis of multi-environmental trials for wheat grain yield based on GGE-biplot analysis under diverse sowing dates. *BMC Plant Biology*, *23*(1), 198.

Sahin, A. W., Wiertz, J., & Arendt, E. K. (2020). Evaluation of a new method to determine the water addition level in gluten-free bread systems. *Journal of Cereal Science*, *93*, 102971.

Saini, P., Kumar, N., Kumar, S., Panghal, A., & Attkan, A. K. (2023). Analysis of engineering properties, milling characteristics, antioxidant potential, and nutritional benefits of purple wheat and its bran. *Food Bioengineering*, *2*(4), 406-419.

Saleh, A. S., Zhang, Q., Chen, J., & Shen, Q. (2013). Millet grains: nutritional quality, processing, and potential health benefits. *Comprehensive Reviews in Food Science and Food Safety*, *12*(3), 281-295.

Sasaki, T., Yasui, T., & Matsuki, J. (2000). Effect of amylose content on gelatinization, retrogradation, and pasting properties of starches from waxy and nonwaxy wheat and their F1 seeds. *Cereal Chemistry*, *77*(1), 58-63.

Satrio, R. D., Fendiyanto, M. H., & Miftahudin, M. (2024). Tools and techniques used at global scale through genomics, transcriptomics, proteomics, and metabolomics to investigate plant stress responses at the molecular level. In *Molecular Dynamics of Plant Stress and its Management* (pp. 555-607). Springer.

Saulnier, L., Sado, P.-E., Branlard, G., Charmet, G., & Guillon, F. (2007). Wheat arabinoxylans: Exploiting variation in amount and composition to develop enhanced varieties. *Journal of Cereal Science*, *46*(3), 261-281.

Saxelby, C. (2005). Low-carb foods: are they a marketing gimmick? *Nutridate*, *16*(2), 1-4.

Schloermann, W., & Glei, M. (2017). Potential health benefits of β-glucan from barley and oat. *Ernahrungs Umschau*, *64*(10), 145-149.

Schnoes, A. M., Ream, D. C., Thorman, A. W., Babbitt, P. C., & Friedberg, I. (2013). Biases in the experimental annotations of protein function and their effect on our understanding of protein function space. *PLOS Computational Biology*, *9*(5), e1003063.

Shapter, F. M., Cross, M., Ablett, G., Malory, S., Chivers, I. H., King, G. J., & Henry, R. J. (2013). High-throughput sequencing and mutagenesis to accelerate the domestication of *Microlaena stipoides* as a new food crop. *PLoS One*, *8*(12), e82641.

Shapter, F. M., Henry, R., & Lee, L. (2008). Endosperm and starch granule morphology in wild cereal relatives. *Plant Genetic Resources*, *6*(2), 85-97.

Sharma, A., Yadav, B. S., & Ritika. (2008). Resistant starch: physiological roles and food applications. *Food Reviews International*, *24*(2), 193-234.

Sharma, C., Pathak, P., Kumar, A., & Gautam, S. (2024). Sustainable regenerative agriculture allied with digital agri-technologies and future perspectives for transforming Indian agriculture. *Environment, Development and Sustainability*, 1-36.

Shen, P., Ha, S. M., Peng, J., Landman, J., & Sagis, L. M. (2025). Role of pulse globulins and albumins in air-water interface and foam stabilization. *Food Hydrocolloids*, *160*, 110792.

Shewry, P. R., & Halford, N. G. (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany*, *53*(370), 947-958.

Shrestha, P. (2020). *Cereal Grains: Physical and Thermal Properties*. Food Tech Notes.

Shukla, K., & Srivastava, S. (2014). Evaluation of finger millet incorporated noodles for nutritive value and glycemic index. *Journal of Food Science and Technology*, *51*(3), 527-534.

Siddiqua, A., Ali, M., & Ahmed, S. (2019). Functional properties of germinated and non-germinated cereals: A comparative study. *Bangladesh Journal of Scientific and Industrial Research*, *54*(4), 383-390.

Singh, J., Dartois, A., & Kaur, L. (2010). Starch digestibility in food matrix: a review. *Trends in Food Science & Technology*, *21*(4), 168-180.

Singh, J., Karmakar, S., & Banerjee, R. (2019). Extraction and nutritional properties of protein derived from rice (*Oryza sativa*) based distillery byproducts: a potential substrate for food formulation. *Biocatalysis and Agricultural Biotechnology*, *22*, 101364.

Sissons, M. (2016). *Durum wheat chemistry and technology*. Academic Press.

Slade, A. J., McGuire, C., Loeffler, D., Mullenberg, J., Skinner, W., Fazio, G., Holm, A., Brandt, K. M., Steine, M. N., & Goodstal, J. F. (2012). Development of high amylose wheat through TILLING. *BMC Plant Biology*, *12*(1), 69.

Smith, M. (2013). *The archaeology of Australia's deserts*. Cambridge University Press.

Smith, R., & Smith, P. A. (2003). An assessment of the composition and nutrient content of an Australian Aboriginal hunter-gatherer diet. *Australian Aboriginal Studies* (2), 39-52.

Sosulski, F. (1962). The centrifuge method for determining flour absorption in hard red spring wheats.

Staacke, T., Mueller-Roeber, B., & Balazadeh, S. (2025). Stress resilience in plants: the complex interplay between heat stress memory and resetting. *New Phytologist*, *245*(6), 2402-2421.

Stacy, R. A., Espelund, M., Sæbøe-Larssen, S., Hollung, K., Helliesen, E., & Jakobsen, K. S. (1995). Evolution of the group 1 late embryogenesis abundant (Lea) genes: analysis of the Lea B19 gene family in barley. *Plant Molecular Biology*, *28*, 1039-1054.

Statista. (2024). *Production volume of oats in Australia from financial year 2011 to 2024, with a forecast for 2025*.

Suri, S., & Singh, A. (2023). Modification of starch by novel and traditional ways: influence on the structure and functional properties. *Sustainable Food Technology*, *1*(3), 348-362.

Tabibloghmany, F. S., & Ehsandoost, E. (2014). Investigation of nutritional and functional properties of resistant starch in food industry: A review. *International Journal of Recent Research and Review*, *1*(1), 27-44.

Tacer-Caba, Z., Nilufer-Erdil, D., & Ai, Y. (2015). Chemical composition of cereals and their products. *Handbook of Food Chemistry*, 301-329.

Teklehaimanot, W. H., & Emmambux, M. N. (2019). Foaming properties of total zein, total kafirin and pre-gelatinized maize starch blends at alkaline pH. *Food Hydrocolloids*, *97*, 105221.

Tester, R. F., & Karkalas, J. (2001). The effects of environmental conditions on the structural features and physico-chemical properties of starches. *Starch-Stärke*, *53*(10), 513-519.

Thermo Fisher Scientific. (n.d). *Coomassie Blue Staining Protocol*.

Thewissen, B. G., Celus, I., Brijs, K., & Delcour, J. A. (2011). Foaming properties of wheat gliadin. *Journal of Agricultural and Food Chemistry*, *59*(4), 1370-1375.

Tungland, B., & Meyer, D. (2002). Nondigestible oligo-and polysaccharides (Dietary Fiber): their physiology and role in human health and food. *Comprehensive Reviews in Food Science and Food Safety*, *1*(3), 90-109.

U.S. Department of Agriculture. (2024). Grain: World Markets and Trade.

U.S. Department of Agriculture, F. A. S. (2023). *AUSTRALIA WHEAT: Production to fall from record highs after extended dryness*.

UniProt Consortium. (2025). *UniProtKB/Swiss-Prot protein knowledgebase release 2025_02 statistics*

United Nations. (2024). *Population Division, 2024. World population prospects 2024: Summary of results*.

Van Amelsvoort, J., & Weststrate, J. A. (1992). Amylose-amylopectin ratio in a meal affects postprandial variables in male volunteers. *The American Journal of Clinical Nutrition*, *55*(3), 712-718.

Van de Vondel, J., Lambrecht, M. A., & Delcour, J. A. (2020). Osborne extractability and chromatographic separation of protein from quinoa (*Chenopodium quinoa* Willd.) wholemeal. *LWT- Food Science and Technology*, *126*, 109321.

Van Toan, N., & Tran, N. T. T. (2012). Preparation and quality evaluation of flour and biscuits made from red corn powder using as supplement with different ratios of red corn flour. *International Journal of Scientific Research and Management (IJSRM)*, *10*(2), 43-62.

Varghese, S., Awana, M., Mondal, D., Rubiya, M., Melethil, K., Singh, A., Krishnan, V., & Thomas, B. (2022). Amylose–Amylopectin Ratio: Comprehensive Understanding of Structure, Physicochemical Attributes, and Applications of Starch. In *Handbook of biopolymers* (pp. 1-30). Springer.

Voconiq. (2023). *Live Exports and the Australian Community: A national survey*. A. Voconiq.

Vujanovic, V., St-Arnaud, M., Barabé, D., & Thibeault, G. (2000). Viability testing of orchid seed and the promotion of colouration and germination. *Annals of Botany*, *86*(1), 79-86.

Wang, C., Tian, Z., Chen, L., Temelli, F., Liu, H., & Wang, Y. (2010). Functionality of barley proteins extracted and fractionated by alkaline and alcohol methods. *Cereal Chemistry*, *87*(6), 597-606.

Wang, L., Wang, J., Wang, J., Guo, Z., Li, Z., Qiu, J., & Wang, L. (2024). Soluble and insoluble dietary fiber at different ratios: Hydration characteristics, rheological properties, and ameliorative effects on constipation. *Food Chemistry: X*, *24*, 101996.

Wang, N. (2020). Development of a method for determining oil absorption capacity in pulse flours and protein materials. *Cereal Chemistry*, *97*(6), 1111-1117.

Wang, S., Chao, C., Cai, J., Niu, B., Copeland, L., & Wang, S. (2020). Starch–lipid and starch–lipid–protein complexes: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, *19*(3), 1056-1079.

Wang, Y., Tu, M., He, G., Li, Y., & Chang, J. (2023). Toward Exploring and Utilizing the Nutritional and Functional Properties of Cereal Crops. *Foods*, *12*(5), 976.

Whan, A. P., Smith, A. B., Cavanagh, C. R., Ral, J.-P. F., Shaw, L. M., Howitt, C. A., & Bischof, L. (2014). GrainScan: a low cost, fast method for grain size and colour measurements. *Plant Methods*, *10*(1), 1-10.

Williams, B. A., Mikkelsen, D., Flanagan, B. M., & Gidley, M. J. (2019). "Dietary fibre": moving beyond the "soluble/insoluble" classification for monogastric nutrition, with an emphasis on humans and pigs. *Journal of Animal Science and Biotechnology*, *10*, 1-12.

Williams, L. B., Birch, J., Zakaria, R., Nguyen, H., Itsiopoulos, C., & Wright, P. F. (2024a). Comparing the nutritional composition and antioxidant properties of an Australian native grain variety with commonly consumed wheat. *International Journal of Food Science & Technology*.

Williams, L. B., Birch, J., Zakaria, R., Nguyen, H., Itsiopoulos, C., & Wright, P. F. (2024b). Comparing the nutritional composition and antioxidant properties of an Australian native grain variety with commonly consumed wheat. *International Journal of Food Science & Technology*, *59*(7), 4939–4948.

Wilman, E. A. (2015). An economic model of aboriginal fire-stick farming. *Australian Journal of Agricultural and Resource Economics*, *59*(1), 39-60.

Woodhouse, M., & Hufford, M. (2019). Parallelism and convergence in post-domestication adaptation in cereal grasses. *Philosophical Transactions of the Royal Society B*, *374*(1777), 20180245.

Wrigley, C. W., Corke, H., Seetharaman, K., & Faubion, J. (2015). *Encyclopedia of food grains*. Academic Press.

Wu, H., Flint, A. J., Qi, Q., Van Dam, R. M., Sampson, L. A., Rimm, E. B., Holmes, M. D., Willett, W. C., Hu, F. B., & Sun, Q. (2015). Association between dietary whole grain intake and risk of mortality: two large prospective studies in US men and women. *JAMA internal medicine*, *175*(3), 373-384.

Wu, Y.-Y., Chou, Y.-R., Wang, C.-S., Tseng, T.-H., Chen, L.-J., & Tzen, J. T. (2010). Different effects on triacylglycerol packaging to oil bodies in transgenic rice seeds by specifically eliminating one of their two oleosin isoforms. *Plant Physiology and Biochemistry*, *48*(2-3), 81-89.

Wurm, P. A., Campbell, L., Batten, G., & Bellairs, S. M. (2012). *Australian Native Rice: A new sustainable wild food enterprise*. RIRDC.

Xu, R., Coventry, D., Farhoodi, A., & Schultz, J. (2002). Soil acidification as influenced by crop rotations, stubble management, and application of nitrogenous fertiliser, Tarlee, South Australia. *Soil Research*, *40*(3), 483-496.

Yu, J., Xia, J., Yang, C., Pan, D., Xu, D., Sun, G., & Xia, H. (2022). Effects of oat beta-glucan intake on lipid profiles in hypercholesterolemic adults: A systematic review and meta-analysis of randomized controlled trials. *Nutrients*, *14*(10), 2043.

Yu, P., Xu, R., Abramson, M. J., Li, S., & Guo, Y. (2020). Bushfires in Australia: a serious health emergency under climate change. *The Lancet Planetary Health*, *4*(1), e7-e8.

Yun, S. H., & Matheson, N. K. (1990). Estimation of amylose content of starches after precipitation of amylopectin by concanavalin-A. *Starch-Stärke*, *42*(8), 302-305.

Zhang, H., Wang, B., Li Liu, D., Zhang, M., Feng, P., Cheng, L., Yu, Q., & Eamus, D. (2019). Impacts of future climate change on water resource availability of eastern Australia: A case study of the Manning River basin. *Journal of hydrology*, *573*, 49-59.

Zhang, M., Viscarra Rossel, R. A., Zhu, Q., Leys, J., Gray, J. M., Yu, Q., & Yang, X. (2022). Dynamic modelling of water and wind erosion in Australia over the past two decades. *Remote Sensing*, *14*(21), 5437.

Zhao, G., Zou, C., Li, K., Wang, K., Li, T., Gao, L., Zhang, X., Wang, H., Yang, Z., & Liu, X. (2017). The Aegilops tauschii genome reveals multiple impacts of transposons. *Nature Plants*, *3*(12), 946-955.

Zhao, J. (2011). Improvement of functionality of barley protein by deamidation.

Zhao, J., Tian, Z., & Chen, L. (2011). Effects of deamidation on aggregation and emulsifying properties of barley glutelin. *Food Chemistry*, *128*(4), 1029-1036.

Zhao, L., Pan, T., Cai, C., Wang, J., & Wei, C. (2016). Application of whole sections of mature cereal seeds to visualize the morphology of endosperm cell and starch and the distribution of storage protein. *Journal of Cereal Science*, *71*, 19-27.

Zhao, M., Xiong, W., Chen, B., Zhu, J., & Wang, L. (2020). Enhancing the solubility and foam ability of rice glutelin by heat treatment at pH12: Insight into protein structure. *Food Hydrocolloids*, *103*, 105626.

Zhao, S., Ren, Y., & Wei, C. (2022). Staining Starch with Iodine Solution. In *Histochemistry of Single Molecules: Methods and Protocols* (pp. 281-290). Humana Press.

Zheng, S. J. (2010). Crop production on acidic soils: overcoming aluminium toxicity and phosphorus deficiency. *Annals of Botany*, *106*(1), 183-184.

Zhu, F. (2018). Relationships between amylopectin internal molecular structure and physicochemical properties of starch. *Trends in Food Science & Technology*, *78*, 234-242.

Zhu, X., Wang, R., Feng, W., Chen, Z., & Wang, T. (2021). Rice glutelins and β-conglycinin or glycinin forming binary structures with different structural and functional properties. *Food Biophysics*, *16*, 532-543.

Appendix

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Studies

Studies

Indigenous Australian grass seeds as grains: macrostructure, microstructure and histochemistry

Farkhondeh Abedi[1], Claudia Keitel[2,3], Ali Khoddami[1,3], Salla Marttila[4], Angela L. Pattison[3,5] and Thomas H. Roberts[*1,3]

[1]School of Life and Environmental Sciences, University of Sydney, Camperdown, NSW 2006, Australia
[2]School of Life and Environmental Sciences, University of Sydney, Camden, NSW 2570, Australia
[3]Sydney Institute of Agriculture, University of Sydney, Camperdown, NSW 2006, Australia
[4]Department of Plant Protection Biology, Resistance Biology Unit, Swedish University of Agricultural Sciences, 23053 Alnarp, Sweden
[5]School of Life and Environmental Sciences, University of Sydney, Narrabri NSW 2390, Australia

[*]Corresponding author's email address: thomas.roberts@sydney.edu.au

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Abstract. Utilization of grains of local grasses by Australia's First Nations people for food and connection to Country has largely been lost due to colonization. Native Australian grain production has the potential to deliver environmental, economic, nutritional and cultural benefits to First Nations people and the wider community. Revitalization of the native grain food system can only be achieved if relevant properties of the grains are elucidated. This study aimed to characterize the grain structure and histochemistry of four Australian native grasses: *Dactyloctenium radulans* (Button Grass), *Astrebla lappacea* (Curly Mitchell Grass), *Panicum decompositum* (Native Millet) and *Microlaena stipoides* (Weeping Grass). For these species, as well as wheat and sorghum, whole-grain images were obtained via stereo microscopy, starch and the embryo were visualized, and sections of fixed grains were imaged via bright-field and fluorescence microscopy. The shape, size and colour of the whole native grains varied between the species. The aleurone layer was one-cell thick in the native species, as in the domesticated grains, except for Weeping Grass, which had a two-cell-thick aleurone. In the native grains, endosperm cell walls appeared thinner than in wheat and sorghum. Starch granules in Button Grass, Curly Mitchell Grass and Native Millet were found mainly in the central region of the starchy endosperm, with very few granules in the sub-aleurone layer, whereas Weeping Grass had abundant starch in the sub-aleurone. Protein appeared most abundant in the aleurone and sub-aleurone layers of the native grains, although in Button Grass, the starchy endosperm was observed to be rich in protein, as in wheat and sorghum. As a proportion of the whole grain, the embryo was larger in the native species than in wheat. The differences found in the grain properties among the four native Australian species have important implications for the agri-food industry in a changing climate.

Keywords: Aleurone; *Astrebla lappacea* (Curly Mitchell Grass); cell wall; *Dactyloctenium radulans* (Button Grass); embryo; endosperm; fluorescence microscopy; histochemistry; *Microlaena stipoides* (Weeping Grass); .*Panicum decompositum* (Native Millet).

Introduction

More than 1100 species of native grasses endemic to Australia have been identified (Penfold and Collins 2012). Seeds of some of these grasses have played an important part in the traditional diet of First Nations people for millennia, depending on the specific region and particular Indigenous culture (Tindale 1977; Pascoe 2014). In arid and semi-arid landscapes in particular, grains from grasses (mixed with seeds from non-grass species) were an important food source; they were processed into flour and cooked (Foster *et al.* 2010; Drake *et al.* 2021). In the Alligator Rivers Region of Northern Australia, it is believed that grains and seeds were likely consumed as early as 65,000 years ago (Clarkson *et al.* 2017; Pattison *et al.* in press). Many of these Australian native grasses are deep-rooted, tolerant to high temperatures and drought and well-integrated into the natural biodiversity of Australia through millions of years of evolution (Pascoe 2014; Chivers *et al.* 2015).

Wheat, rice, maize, barley, rye and oats were brought to Australia by English settlers in 1788 (Redden *et al.* 2020). Since then, almost all grain production in Australia has been from these and other annual cereal crops, such as sorghum. The systems used for cereal production in Australia have provided abundant food, feed and export income to the nation, but they have also been associated with environmental problems including soil erosion and degradation, salinity and loss of nutrients, challenging their sustainability (Hatton and Nulsen 1999; Bell *et al.* 2010; Redden *et al.* 2020).

Average wheat yields in northwest New South Wales (NSW) have typically ranged from 3 to 4 tonnes per hectare (t/ha) in recent decades. For the same region, however, estimated native grains yields vary between 0.1 and 0.5 t/ha, depending on factors such as species, rainfall patterns and management practices (Sydney Institute of Agriculture 2020). In at least some regions of Australia, native perennial grain cropping has the potential to involve less soil disturbance and

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greater resilience to drought, thus expanding current cereal production zones to marginal lands not suitable for annual crops including wheat and sorghum (Glover and Reganold 2010). Larger and deeper root systems in perennial grasses absorb more water than annuals, decreasing deep drainage, nitrate losses and acidification rates. The potential of perennial grasses to provide year-round ground cover also plays an important role in decreasing soil erosion (Edwards *et al.* 2015). Native grain production provides promising opportunities for commercial food applications and the involvement of Indigenous communities, which have to date been grossly underrepresented in the Indigenous foods market (Drake *et al.* 2021).

A greater understanding of the biology of candidate Australian native grasses for commercial grain production is required to promote and develop a viable native grains industry. Grass inflorescences are composed of spikelets, which themselves contain 1–40 florets. Each spikelet consists of two small sterile bracts (the glumes), which surround the florets. Each floret comprises the lemma (the outer bract), palea (inner bract) and lodicule. The palea and lemma are considered to correspond to a prophyll and a bract, respectively, whereas the lodicule contributes to opening the florets and corresponds to the petals in eudicots (Jackson and Jacobs 1985; Schmidt and Ambrose 1998; Bommert *et al.* 2005; Jacobs *et al.* 2008; Ciaffi *et al.* 2011).

The fruit of a grass is a caryopsis, which holds a single seed considered the greater part of the mature fruit. The seed consists of the embryo, endosperm, nucellus, testa (seed coat) and fruit coat (pericarp) firmly bound to the testa (Evers *et al.* 1999). The embryo is formed from an embryonic axis, consisting of the plumule and radicle, and the scutellum, which is a shield-like structure, homologous to a cotyledon, placed between the embryonic axis and endosperm. The scutellum transfers nutrients from the endosperm to the embryo (Evers and Millar 2002). The endosperm is the major storage tissue of the caryopsis and is made up of two sub-tissues: the starchy endosperm and the aleurone. Both the starch of the endosperm and the storage proteins that enclose the starch are hydrolysed by enzymes to feed the embryo upon germination. Layers of aleurone cells surround the starchy endosperm and produce these enzymes required for this mobilization (Evers and Millar 2002; Antonini *et al.* 2018).

The identity, proportions and position of the major storage components of the grain and their distribution and interactions at finer scales play an important role in determining the nutritional and processing properties of the grain and flour (Irving and Jideani 1997). The microstructure of plant-based foods, such as the degree of integrity of cell walls, can influence the digestion of dietary macronutrients. Starch, proteins and lipids are less susceptible to enzyme attack during digestion when encapsulated inside cell walls (Bhattarai *et al.* 2018).

The economic cost of grain processing is a key determinant of whether a particular species can be profitable in contemporary markets, but the amount of time and energy consumed during grain processing is easily overlooked in gauging the commercial potential of native grains (Sydney Institute of Agriculture 2020). To better understand the opportunities and constraints associated with commercial native grain production, key physicochemical properties of the grains require investigation.

This study examined the microstructure and histochemistry of the grains of four Australian native grasses: *Dactyloctenium radulans* (Button Grass), *Astrebla lappacea* (Curly Mitchell Grass), *Panicum decompositum* (Native Millet), and *Microlaena stipoides* (Weeping Grass). These species were selected based on their yield potential in their natural range, threshing simplicity (Pattison *et al.* 2023), their importance as food sources for Gamilaroi people via workshop discussions (McKemey and White 2011), and potential for commercial food applications. The potential for Weeping Grass to become a perennial grain crop in Australia has been investigated through research begun in the early 2000s (Davies *et al.* 2005; Shapter and Chivers 2015). In the former study, grain yield and its components were measured in 46 accessions of *Microlaena stipoides*. The authors found a high degree of variability among the accessions, including a 20-fold range in grain yield and a 5-fold range in grain weight. In the latter study, the productivity of nine *M. stipoides* ecotypes was assessed for grain production when grown as a companion plant with coffee or olives.

For the four native grass species listed above, the aims of this study were to analyse grain dimensions, localize and image the embryo, endosperm and aleurone layer, and determine the distribution of starch, protein and β-glucan in the grains using bright-field and fluorescence microscopy. Another Australian native grass species that was used by First Nations people as food and that has been analysed in a similar way to the current study is Kangaroo Grass (*Themeda triandra*) (Cowley *et al.* 2023); thus, we make several comparisons to Kangaroo Grass in this study. Our results were expected to link traditional knowledge of food properties of the grains with physical features at a microscopic level, explain and contextualize nutritional information associated with these grains, and provide a foundation for future research on specific food applications of these grains.

Materials and Methods

Plant materials

Australian native grains (mature seeds) selected for this study were from Button Grass (*Dactyloctenium radulans*), Curly Mitchell Grass (*Astrebla lappacea*), Native Millet (*Panicum decompositum*) and Weeping Grass (*Microlaena stipoides*) (Table 1). Sorghum (*Sorghum bicolor* cv. Buster) and wheat (*Triticum aestivum*) grains were used as domesticated comparator grains. The grains from Button Grass and Native Millet were harvested by hand from around 100 plants in each case, and the seeds pooled.

Button Grass and Native Millet seeds were collected from the University of Sydney Plant Breeding Institute and Llara farm, both located in Narrabri (latitude: −30°18′60″ S; longitude: 149°45′60″ E) in northern NSW, Australia. Curly Mitchell Grass seeds were obtained from Thallon (latitude: −28°38′60″S; longitude: 148°51′60″E), Queensland and Weeping Grass seeds were supplied by Creative Native Food Service Co, Australia, harvested from a farm outside of Armidale (latitude: −30°30′30″ S; longitude: 151°40′16″E), NSW in May 2021. Seeds were threshed with methods best suited to each species (Pattison *et al.* in press), cleaned manually by sifting and winnowing to remove impurities such as chaff and dust and maintained at 4 °C until analysis.

Table 1. Scientific names, basic properties and distribution in Australia of the native grain species used in this study.

Species	Scientific name	C3/C4	Plant height (cm)	Spikelet length (mm)	Distribution in Australia
Button Grass [1, 2, 3]	*Dactyloctenium radulans*	C4	Up to 20	5	All states
Curly Mitchell Grass [1,2]	*Astrebla lappacea*	C4	Up to 100	7–13	All states except Victoria
Native Millet [2, 3, 4, 5]	*Panicum decompositum*	C4	Up to 100	2.5–3.5	All states
Weeping Grass [1, 2, 6]	*Microlaena stipoides*	C3	10–100	10–40	Eastern and Southern Australia

[1] Cavanagh *et al.* (2019).
[2] Jacobs *et al.* (2008).
[3] Rose and Rose (2012).
[4] Edwards *et al.* (2015).
[5] Turner (1895).
[6] Lamp *et al.* (1990).

Grain shape, size and weight distribution

For external morphology analysis, images of the whole grains were taken using a Leica MZ16FA fluorescence stereomicroscope (Australian Centre for Microscopy and Microanalysis, University of Sydney). Microscopy images of 20 grains of each native species were used to determine grain lengths and widths using Fiji ImageJ ver1.54c. Weights of 20 seeds of each species were measured using an analytical balance with a readability of five decimal places.

Grain microstructure and histochemistry

Grains were fixed in a 4 % formaldehyde solution at 4 °C for 24 h, sectioned longitudinally to 2–3 mm thickness and fixed again overnight. The sections were washed in distilled water (3 × 30 min) and dehydrated with an increasing ethanol series: 30 %, 50 %, 70 % and 90 % (30 min each), then with 100 % ethanol (3 × 30 min). Samples were then infiltrated with LR White resin at 25 %, 50 %, 75 % (12 h each) and 100 % (2 × 12 h). Samples were then positioned in Beem embedding capsules, filled with 100 % LR White resin to the top and polymerized at 60 °C (48 h) (Zhao *et al.* 2016).

Longitudinal sections (1 μm) were prepared with a Leica EM UC7 ultramicrotome using a Diatome histo diamond knife and collected on microscope slides. For bright-field microscopy, sections were stained with 0.1 % methylene blue for 2 min to observe cell walls (blue) and starch (white). For fluorescence microscopy, sections were stained with 0.1 % acid fuchsin for 4 min, which stains protein red, followed by staining with 0.01 % calcofluor white for 1 min, which stains cell walls blue if they contain β-glucan or cellulose (Nicholas *et al.* 1994; Kamal-Eldin *et al.* 2009; Herburger and Holzinger 2016). After each staining, sections were washed under running tap water for 1 min and dried at room temperature. The sections were mounted with one drop of glycerol, a coverslip added, and imaged using an Olympus VS120 slide scanner at 40× magnification. The images selected for the figures in the manuscript were based on intactness of the sectioned tissue, a lack of shrinking or other damage, and consistent staining over the whole section.

Starch distribution

Grains were cut transversely using a scalpel. The sections were suspended in 25 % Lugol's iodine solution (30 s) at room temperature and observed under a Leica M125 C stereo microscope. Starch stained with Lugol's iodine turned black immediately (Zhao *et al.* 2022).

Visualization of the embryo

The embryo of each of the native grains was visualized using vital staining. Grains were cut longitudinally through the embryo with a scalpel and then immersed in 1 % (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) solution and placed in the dark for 2 h at room temperature. Microscopy images were taken using a Leica M125 C stereo microscope. Viable embryos turned orange to bright red (Vujanovic *et al.* 2000).

Results and Discussion

Whole-grain shape, size, weight and colour

The shape, colour and size of whole grains, imaged from four angles to highlight different structures, varied between the four native grain species (Figs 1 and 2; Supporting Information—Table S1). Button Grass grains had an ovoid shape, whereas Native Millet grains tended to be long and ellipsoid. Curly Mitchell Grass and Weeping Grass grains were found to be pyriform and canoe-shaped, respectively (Fig. 1). The smallest and largest (and heaviest) grains among the species were those of Button Grass and Weeping Grass, respectively, the latter being highly elongated (Fig. 2), like that of Kangaroo Grass (Cowley *et al.* 2023). The colour of the seeds varied from yellow-brown in Curly Mitchell Grass, orange-brown in Button Grass and dark brown in Weeping Grass to shiny dark brown or black in Native Millet.

The shiny appearance of the Native Millet was due to the retention of the lemma and palea, despite applying the most effective threshing method. Fig. 1C reveals how tight and far the lemma is wrapped around the palea, and thus why these structures are so difficult to remove. Historically, it was common for Indigenous people to incorporate the lemma and palea of Native Millet grain into the final flour (Latz 1995). In oat grain, this results in an increase in the fibre and a decrease in digestible carbohydrate content (Ganssmann and Vorwerck 1995). The Cooper Creek people of Central Australia used two stones—a large, uneven slab and a smaller, ball-shaped one—to grind the whole seeds of Native Millet into a meal, as reported by Gregory in 1887. The seeds were placed on the larger stone and then ground into a meal, sometimes using water to aid in the process (Tindale 1977; Clarke 2011).

Native Millet seeds can be ground, with the resulting flour absorbing water (i.e. caking) readily. However, grinding Native Millet seeds is more difficult than for seeds of other species that are both soft and readily fractured (Mildwaters and Clarkson 2020). In a study by Jenifer *et al.* (2023), Native

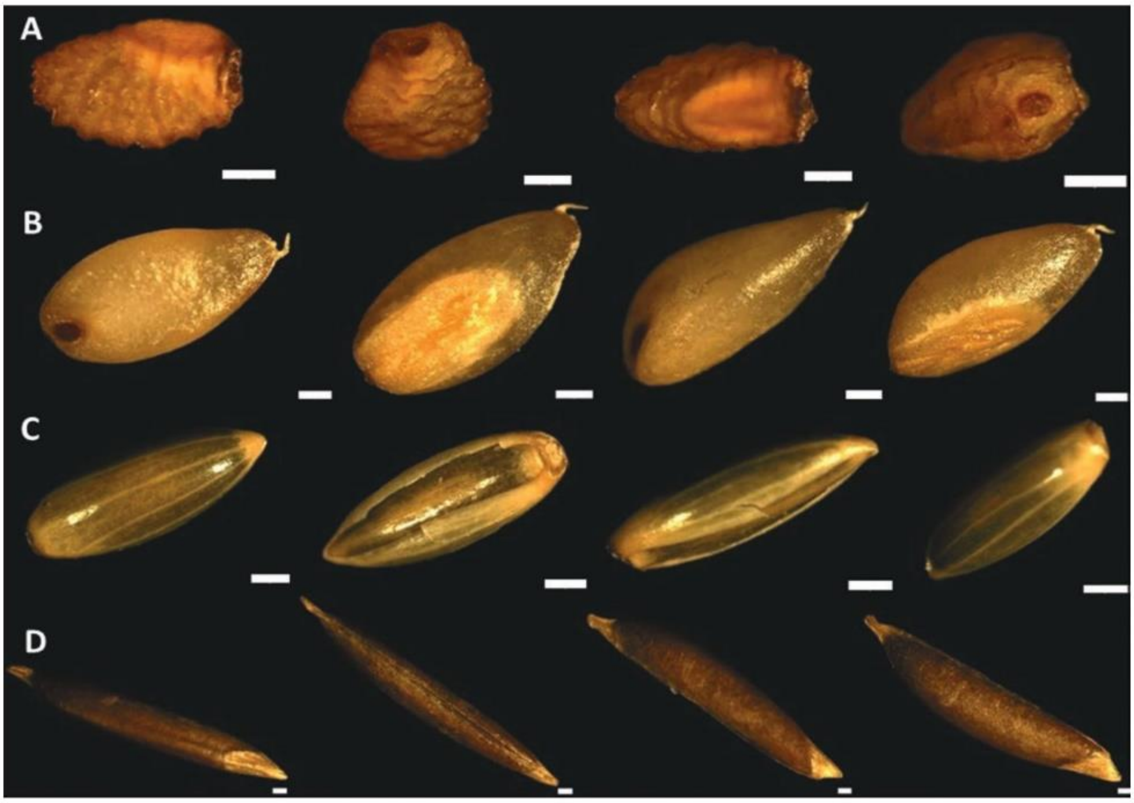

Figure 1. External appearance of the native grains. Images of the whole grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet and (D) Weeping Grass from different angles using a Leica MZ16FA stereomicroscope (scale bar = 100 μm). Note the shiny and stripey palea and lemma surrounding the Native Millet grain.

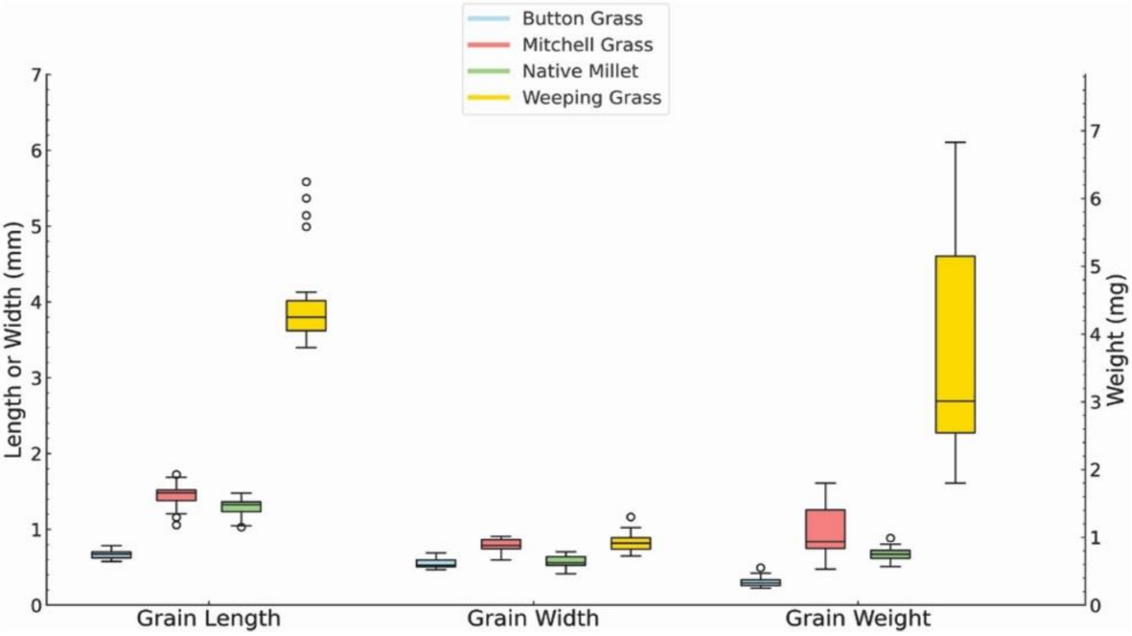

Figure 2. Box plot analysis of grain length, width and weight of the four native grasses ($n = 20$). Length and width (in mm) of the grains were determined using a Leica MZ16FA stereomicroscope and FIJI ImageJ software. Weight of the grains was measured using an analytical balance with a readability of five decimal places. Each box plot represents the distribution of values for Button Grass, Curly Mitchell Grass, Native Millet and Weeping Grass, respectively. The horizontal line within each box indicates the median value, while the upper and lower borders show the interquartile range (25th to 75th percentiles). Whiskers extend to the 5th and 95th percentiles. Points outside the whiskers are outliers.

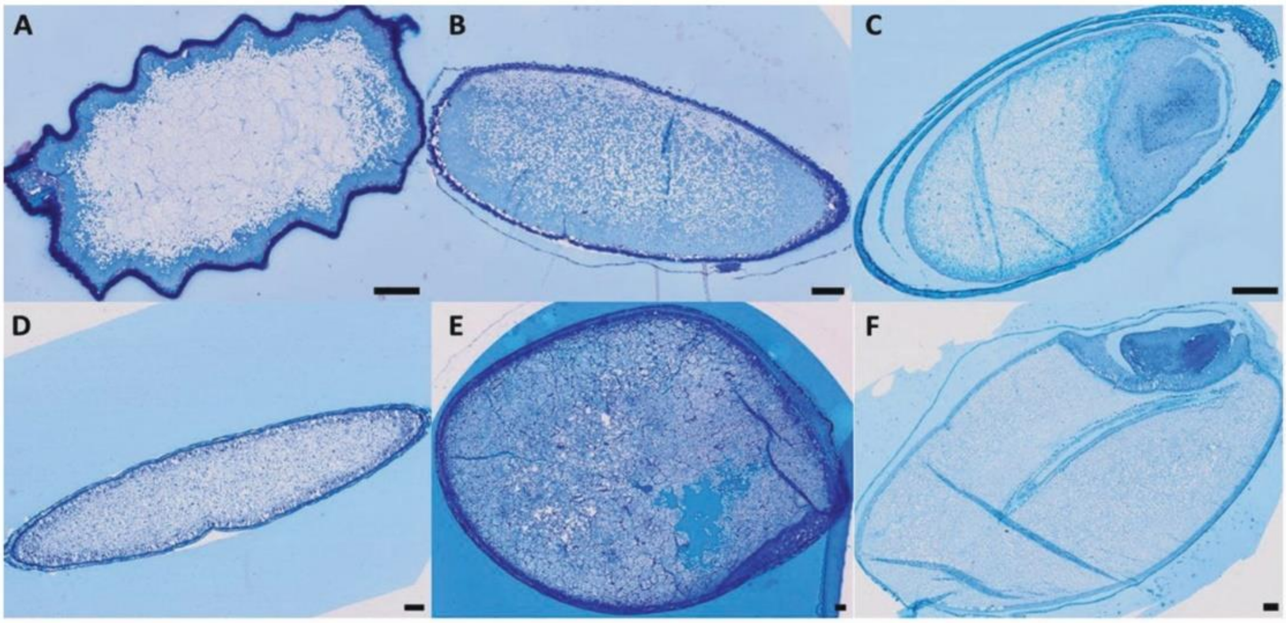

Figure 4. Tissue structure of the native and domesticated grains. Resin-embedded grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, (D) Weeping Grass (E) sorghum and (F) wheat were sectioned longitudinally (1 µm) with an ultramicrotome and stained with 0.1 % methylene blue for 2 min. The tissue structure (stained) was observed using an Olympus VS120 slide scanner at 40× magnification (scale bar = 100 µm). Starch appears white, whereas cell walls (specifically beta-glucan and cellulose) appear stained. Note the palea and lemma surrounding the Native Millet seed (C).

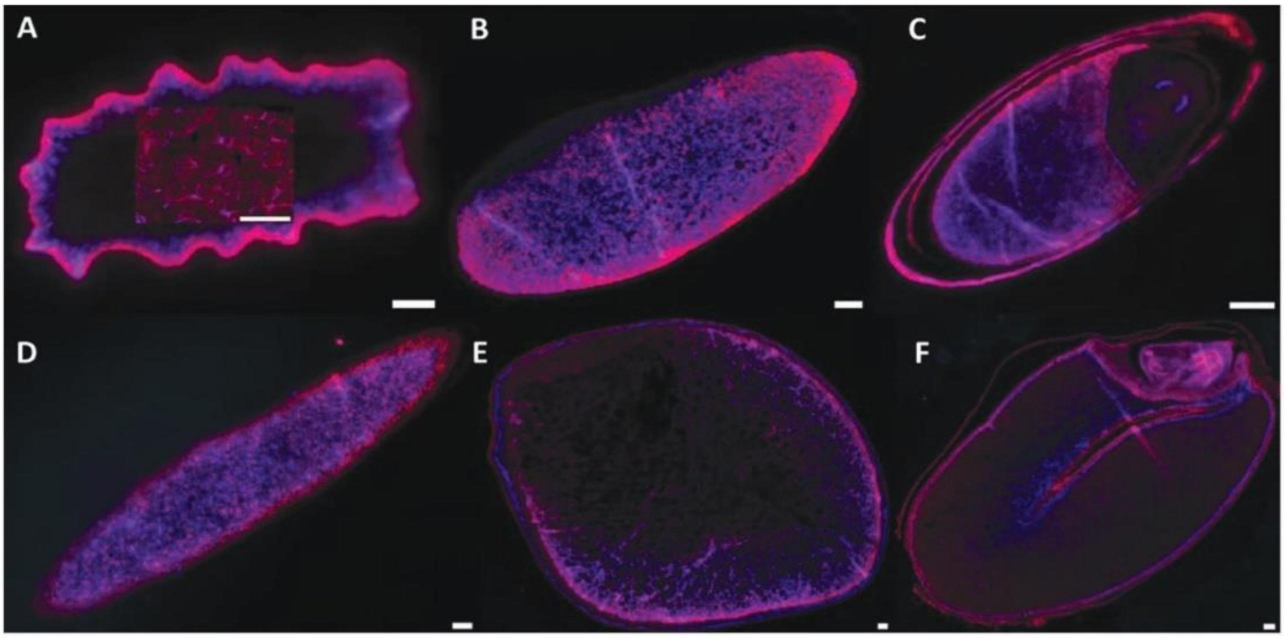

Figure 5. Protein and cell wall visualization of the native and domesticated grains. Resin-embedded grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, (D) Weeping Grass, (E) sorghum and (F) wheat were sectioned longitudinally (1 µm) with an ultramicrotome and stained with 0.1 % acid fuchsin for 4 min and 0.01 % calcofluor white for 1 min. The staining showing proteins in red and cell walls (containing β-glucan or cellulose) blue, respectively, was observed using an Olympus VS120 slide scanner at 40× magnification (scale bar = 100 µm). The section in the box in the middle of the Button Grass image shows the endosperm structure at a higher exposure setting but at the same magnification. Note the palea and lemma surrounding the Native Millet seed (C). For colour figures, refer to the online version.

β-glucan in appropriate concentrations and a decreased likelihood of chronic health issues. By reducing blood serum cholesterol levels, β-glucan helps mitigate the risks associated with cardiovascular disease (Braaten *et al.* 1994). Additionally, it aids in regulating blood glucose levels, thereby contributing to the management of diabetes (Wood *et al.* 1994).

Although grains of grasses generally have low cellulose contents (Fincher and Stone 2004), and thus high β-glucan

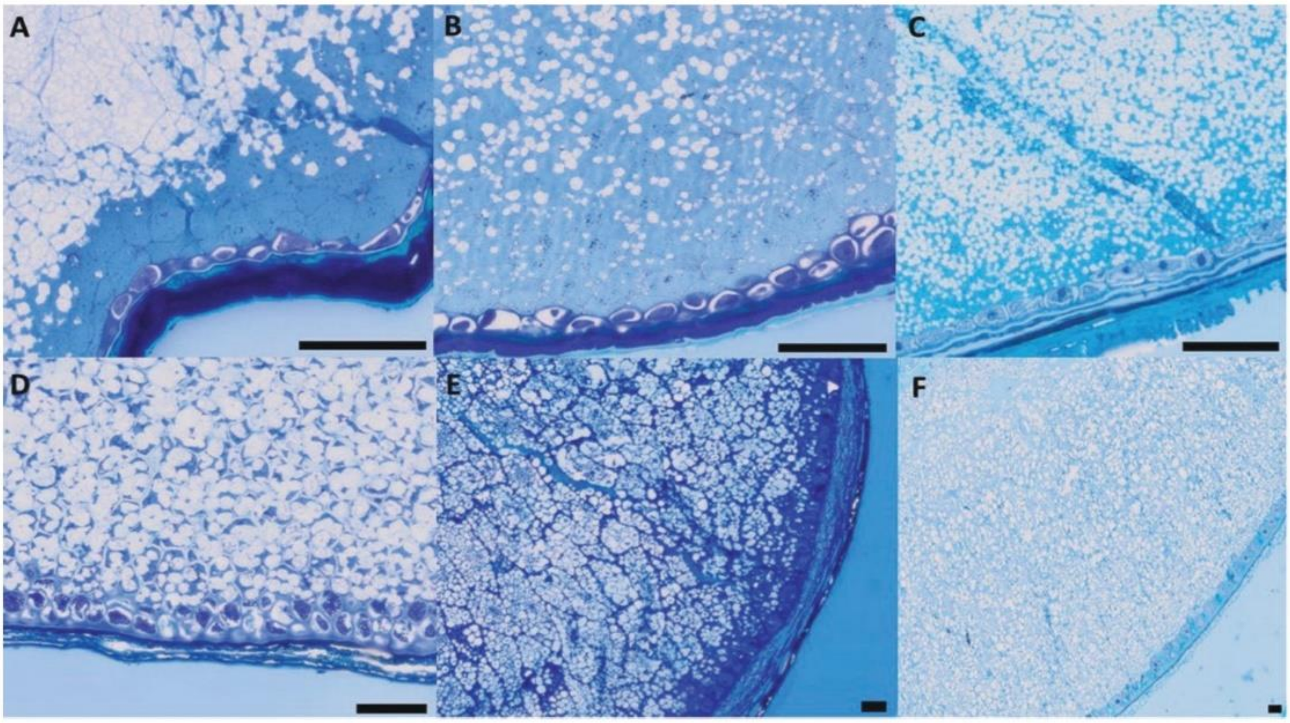

Figure 6. Detailed structure of the aleurone layer and starchy endosperm of the native and domesticated grains. Close-up microscopy images (based on images from Fig. 4) of longitudinal sections (1 μm) of the resin-embedded grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, (D) Weepin Grass, (E) sorghum and (F) wheat, stained with 0.1 % methylene blue for 2 min. The staining showing the aleurone and endosperm cell structure of the grains was visualized using an Olympus VS120 slide scanner at 40× magnification (scale bar = 50 μm).

in the endosperm is the more likely explanation for the observations in Fig. 5, there is an interesting exception in rice, where endosperm cell walls of mature rice grains contain significant amounts (up to 30 %) of cellulose (Shibuya and Nakane 1984).

Based on the observed red or pink staining in sections stained with acid fuchsin (Fig. 5), endosperm protein was most concentrated in the aleurone and sub-aleurone layers of all the native grains, except Button Grass, in which the starchy endosperm appeared to be rich in protein, as in wheat and sorghum grain. In Button Grass, the endosperm cell walls were stained a mixture of blue and pink, suggesting the presence of both protein and β-glucan (Fig. 5A). The red stain of the aleurone cells in all species indicated an abundance of protein in that tissue (Fig. 5).

Aleurone layer microstructure

Cells in the aleurone layer produce enzymes required for mobilization of the endosperm during seed germination (Evers and Millar 2002; Antonini *et al.* 2018) and have significant implications for the nutritional properties and shelf life of the flour. The wheat aleurone layer is highly nutritious as it contains abundant dietary fibre as well as several classes of biologically active compounds, including phenolic antioxidants, phytate, lipids and vitamins B and E. Furthermore, the dietary fibre present in the wheat aleurone layer is classified as a non-starch polysaccharide, playing a significant role in influencing the properties of starch-based foods, such as their rheology and texture. Nevertheless, the oxidative rancidity of wheat flour containing substantial aleurone tissue poses a significant challenge during storage and production, resulting in a shortened shelf life (Jin *et al.* 2021).

The thickness of the aleurone layer (i.e. the number of adjacent cells), as well as the size and shape of the aleurone cells, varied among the native species (Fig. 6). The aleurone layer was 1–2 cells thick in Weeping Grass (Fig. 6) (Kasem *et al.* 2011), but only one cell thick in the other native species, as in Kangaroo Grass (Cowley *et al.* 2023), as well as in wheat and sorghum grain (Fig. 6). In barley, the aleurone is 2–4 cells thick (Paleg and Hyde 1964; Clutterbuck and Briggs 1973). The aleurone cells in Weeping Grass appeared to be larger compared to those of the other native species and domesticated grains (Fig. 6D). The corresponding cells in Native Millet (Fig. 6C) were more block-shaped, like those in sorghum (Fig. 6E) and wheat (Fig. 6F), compared to the other native species (Fig. 6A, B and D).

Embryo size and position

With sections of the native grains stained with methylene blue or acid fuchsin/calcofluor white (Figs 4C and 5C), the embryo—including the plumule, radicle and scutellum—was observed only in Native Millet, which was estimated to comprise almost 40 % of the caryopsis, a larger percentage than that for wheat (Figs 4F and 5F). Based on TTC staining of longitudinally cut whole grains, the embryo of Button Grass was located closer to the edge of the grain than the embryos of the other species (including sorghum and wheat) and orientated in parallel to the edge; it was also at the top of the long edge rather than central to the shorter edge (Fig. 7A). The embryos of the other species were at the pole of the grain (Fig. 7B, C, E and F). In Weeping Grass, the embryo did not stain

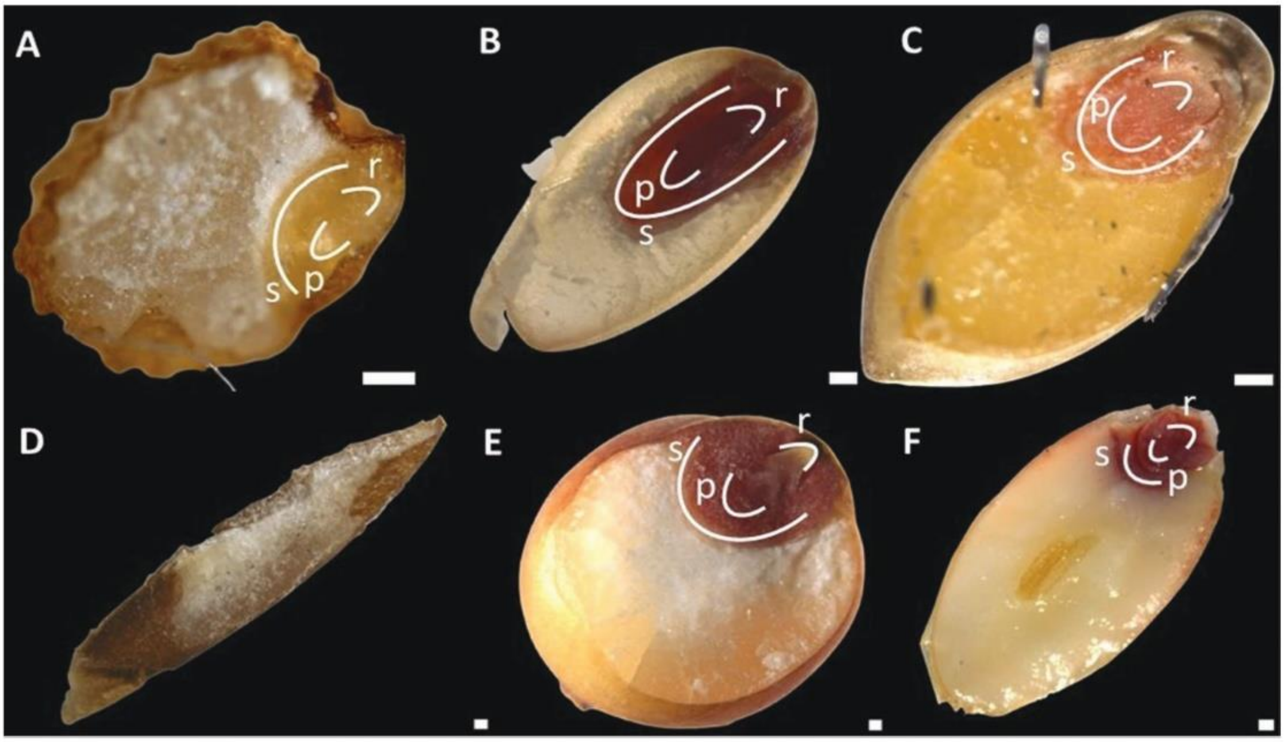

Figure 7. Visualization of the embryo in native and domesticated grains. Grains of (A) Button Grass, (B) Curly Mitchell Grass, (C) Native Millet, (D) Weeping Grass, (E) sorghum and (F) wheat were cut longitudinally through the embryo using a scalpel and stained with 1 % (w/v) TTC solution in the dark for 2 h at room temperature. The grains were observed with a Leica M125 C stereo microscope, showing the embryo structure, size and location. TTC stained the viable embryos orange to bright red. The images illustrate the radicle (r), plumule (p) and scutellum (s) (scale bar = 100 μm). The location of these structures in the Weeping Grass grains could not be determined.

(~10 seeds were tested), suggesting that the seed may have been unviable (Fig. 7D), but further viability testing will be required to clarify this tentative conclusion.

Curly Mitchell Grass appeared to have the largest embryo among the native and domesticated species, constituting almost half of the grain (Fig. 7B). The health benefits of consuming foods made from grains of *Astrebla* spp. were well known to Gamilaroi, Yuwaalaraay and Yuwaaliyaay people and this knowledge was maintained within their language (Pattison *et al.* in press). The large embryo relative to the size of the whole grain of Curly Mitchell Grass (Fig. 7B) may make the flour relatively susceptible to rancidity (as embryos have a high lipid content), like in pearl millet flour, which also contains a large embryo (Nantanga *et al.* 2008).

Conclusions

There are substantial environmental, health and cultural values among Australian First Nations people attached to the revitalization of native grain systems and building pathways for the use of native grains in modern food industries. Knowledge of the structure and composition of grain components is essential for developing industrial processes for grains. This information can complement and highlight the value of traditional knowledge when applied with respect and collaboration with traditional knowledge holders. The macrostructure, microstructure and histochemistry of the native grains reported add to previous work on nutritional composition and thus contribute to creating a basis for further research into food applications of these grains. By complementing chemical analysis, our microscopy data have aided in comprehending and visually representing structural differences in the grains of Button Grass, Curly Mitchell Grass, Native Millet and Weeping Grass, all native grasses of great importance to many First Nations peoples, including the Gamilaroi people of northern NSW. This type of analysis is applicable to other native grains in Australia (Cowley *et al.* 2023) and in other countries.

Our microscopy analysis of the four selected Australian native grain species and the localization of specific macronutrients within the caryopsis should stimulate further research into the connections between the structural, processing and nutritional properties of these grains. Specific considerations include the size and location of the embryo, the distribution of starch, β-glucan/cellulose, protein and the thickness of the aleurone layer.

Supporting Information

The following additional information is available in the online version of this article –

Table S1. Grain length, width and weight for Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass (n = 20; data were used to generate Fig. 2).

Acknowledgements

We acknowledge the Gamilaroi community for sharing their traditional knowledge of native grains. We thank Gamilaroi

Penfold MC, Collins C. 2012. *Native cover crops in viticulture*. Adelaide: Grape and Wine Research and Development Corporation.

Redden R, Norton S, Whitehouse K, Slater A. 2020. History of crop introductions, breeding and selection in Australia: the 140 years from 1788. *Agricultural Science* 31:92–99.

Rose H, Rose C. 2012. *Grasses of coastal NSW*, 1st edn. Sydney: NSW Department of Primary Industries.

Schmidt RJ, Ambrose BA. 1998. The blooming of grass flower development. *Current Opinion in Plant Biology* 1:60–67.

Shapter FM, Chivers IH. 2015. *Commercialisation of elite lines of Microlaena stipoides as a perennial grain*. Canberra, ACT, Australia: Rural Industries Research and Development Corporation.

Shapter FM, Henry R, Lee L. 2008. Endosperm and starch granule morphology in wild cereal relatives. *Plant Genetic Resources* 6:85–97.

Shibuya N, Nakane R. 1984. Pectic polysaccharides of rice endosperm cell walls. *Phytochemistry* 23:1425–1429.

Sydney Institute of Agriculture. 2020. *Native grains from paddock to plate*. Sydney: The University of Sydney.

Tindale NB. 1977. Adaptive significance of the Panara or grass seed culture of Australia. In: Wright RVS, ed. *StoneTools as cultural markers*. Atlantic Highlands, NJ, USA: Humanities Press.

Turner F. 1895. *Australian grasses*. Sydney: Government Printer.

Vasanthan T, Temelli F. 2008. Grain fractionation technologies for cereal beta-glucan concentration. *Food Research International* 41:876–881.

Vujanovic V, St-Arnaud M, Barabé D, Thibeault G. 2000. Viability testing of orchid seed and the promotion of colouration and germination. *Annals of Botany* 86:79–86.

Wang S, Copeland L. 2013. Molecular disassembly of starch granules during gelatinization and its effect on starch digestibility: a review. *Food & Function* 4:1564–1580.

Wood PJ, Braaten JT, Scott FW, Riedel KD, Wolynetz MS, Collins MW. 1994. Effect of dose and modification of viscous properties of oat gum on plasma glucose and insulin following an oral glucose load. *The British Journal of Nutrition* 72:731–743.

Zhao L, Pan T, Cai C, Wang J, Wei C. 2016. Application of whole sections of mature cereal seeds to visualize the morphology of endosperm cell and starch and the distribution of storage protein. *Journal of Cereal Science* 71:19–27.

Zhao S, Ren Y, Wei C. 2022. Staining starch with iodine solution. In: Pellicciari C, Biggiogera M, eds. *Histochemistry of single molecules: methods and protocols*. Totoya, NJ, USA: Springer, 281–290.

Appendix Table 2.1. Individual grain length measurements (mm) for 20 grains from each of the four native grass species: Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass.

	Button Grass (mm)	**Mitchell Grass (mm)**	**Native Millet (mm)**	**Weeping Grass (mm)**
	0.58	1.48	1.24	3.86
	0.60	1.06	1.03	3.90
	0.60	1.16	1.05	3.58
	0.61	1.21	1.12	3.74
	0.62	1.33	1.16	3.82
	0.63	1.36	1.21	3.78
	0.63	1.39	1.26	3.54
	0.63	1.41	1.27	3.64
	0.66	1.44	1.28	3.52
	0.67	1.45	1.32	3.94
	0.68	1.48	1.33	3.98
	0.68	1.50	1.33	3.66
	0.69	1.50	1.33	3.70
	0.69	1.52	1.33	4.13
	0.70	1.52	1.36	3.40
	0.71	1.52	1.37	3.58
	0.74	1.55	1.39	5.14
	0.75	1.63	1.40	5.58
	0.77	1.69	1.40	5.37
	0.78	1.72	1.48	4.99
Mean	**0.67**	**1.44**	**1.28**	**4.04**
SD	**0.06**	**0.17**	**0.12**	**0.66**
CV	**0.09**	**0.11**	**0.09**	**0.16**
CV%	**8.85**	**11.46**	**9.34**	**16.39**
Median	**0.67**	**1.48**	**1.32**	**3.80**

Appendix Table 2.2. Individual grain width measurements (mm) for 20 grains from each of the four native grass species: Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass.

	Button Grass (mm)	**Mitchell Grass (mm)**	**Native Millet (mm)**	**Weeping Grass (mm)**
	0.47	0.75	0.69	0.82
	0.47	0.60	0.41	0.65
	0.47	0.61	0.46	0.71
	0.49	0.65	0.48	0.73
	0.50	0.70	0.49	0.73
	0.51	0.74	0.51	0.73
	0.51	0.75	0.53	0.74
	0.52	0.76	0.54	0.76
	0.52	0.77	0.54	0.76
	0.52	0.78	0.54	0.77
	0.53	0.79	0.56	0.81
	0.55	0.80	0.57	0.83
	0.55	0.82	0.57	0.83
	0.55	0.85	0.58	0.87
	0.59	0.86	0.62	0.87
	0.63	0.86	0.63	0.94
	0.64	0.87	0.64	1.00
	0.66	0.88	0.66	1.01
	0.67	0.90	0.69	1.02
	0.69	0.91	0.70	1.16
Mean	**0.55**	**0.78**	**0.57**	**0.84**
SD	**0.07**	**0.09**	**0.08**	**0.13**
CV	**0.13**	**0.12**	**0.14**	**0.16**
CV%	**12.72**	**11.90**	**14.26**	**15.54**
Median	**0.53**	**0.79**	**0.56**	**0.82**

Appendix Table 2.3. Individual grain weight measurements (mg) for 20 grains from each of the four native grass species: Button Grass, Curly Mitchell Grass, Native Millet, and Weeping Grass.

	Button Grass (mg)	**Mitchell Grass(mg)**	**Native Millet (mg)**	**Weeping Grass (mg)**
	0.25	0.53	0.57	1.80
	0.26	0.72	0.58	1.84
	0.28	0.76	0.60	1.86
	0.28	0.82	0.62	2.05
	0.28	0.83	0.66	2.37
	0.29	0.84	0.70	2.60
	0.29	0.85	0.72	2.68
	0.31	0.85	0.74	2.82
	0.31	0.87	0.75	2.85
	0.32	0.87	0.75	2.91
	0.33	1.00	0.75	3.11
	0.34	1.07	0.75	3.33
	0.35	1.27	0.76	3.61
	0.35	1.29	0.78	4.46
	0.36	1.39	0.80	4.93
	0.41	1.45	0.83	5.80
	0.41	1.47	0.83	5.90
	0.44	1.66	0.84	5.98
	0.47	1.67	0.90	6.53
	0.55	1.80	0.99	6.83
Mean	**0.34**	**1.10**	**0.75**	**3.71**
SD	**0.08**	**0.37**	**0.11**	**1.69**
CV	**0.22**	**0.34**	**0.14**	**0.45**
CV%	**22.49**	**33.50**	**14.31**	**45.47**
Median	**0.33**	**0.94**	**0.75**	**3.01**

Appendix Table 5.1. Concentrations of Osborne protein fractions (albumin, globulin, prolamin, and glutelin) extracted from Button Grass across three biological replicates. Each replicate includes four technical extractions. Protein concentrations are expressed in mg/mL and converted to mg/g of sample. Reported values include individual extraction results, replicate means, and overall mean concentrations (mg/mL and mg/g) for each protein fraction.

	Albumin		**Globulin**		**prolamin**		**Glutelin**	
	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)
Replicate 1	1	0.85	1	0.09	1	1.24	1	1.99
	2	0.19	2	0.07	2	1.14	2	1.10
	3	0.15	3	0.03	3	1.06	3	0.88
	4	0.12	4	0.02	4	0.78	4	0.85
Average (mg/ml)	**0.32**		**0.05**		**1.05**		**1.21**	
Average (mg/g)	**14.87**		**2.28**		**48.12**		**54.96**	
Replicate 2	1	0.95	1	0.13	1	1.85	1	2.00
	2	0.40	2	0.11	2	1.81	2	1.65
	3	0.31	3	0.09	3	1.56	3	1.56
	4	0.25	4	0.08	4	1.40	4	1.52
Average (mg/ml)	**0.47**		**0.10**		**1.66**		**1.68**	
Average (mg/g)	**21.66**		**4.65**		**75.48**		**76.67**	
Replicate 3	1	0.94	1	0.08	1	1.21	1	1.82
	2	0.21	2	0.05	2	1.30	2	1.10
	3	0.15	3	0.03	3	1.05	3	0.93
	4	0.15	4	0.02	4	0.96	4	0.82
Average (mg/ml)	**0.36**		**0.05**		**1.13**		**1.17**	
Average (mg/g)	**16.65**		**2.098**		**51.54**		**53.32**	
Mean (mg/ml, n = 3)	**0.39**		**0.07**		**1.28**		**1.35**	
Mean (mg/g, n = 3)	**17.74**		**3.1**		**58.38**		**61.66**	

Appendix Table 5.2. Concentrations of Osborne protein fractions (albumin, globulin, prolamin, and glutelin) extracted from Curly Mitchell Grass across three biological replicates. Each replicate includes four technical extractions. Protein concentrations are expressed in mg/mL and converted to mg/g of sample. Reported values include individual extraction results, replicate means, and overall mean concentrations (mg/mL and mg/g) for each protein fraction.

	Albumin		**Globulin**		**prolamin**		**Glutelin**	
	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)
Replicate 1	1	4.61	1	1.47	1	1.64	1	4.63
	2	1.69	2	0.58	2	0.72	2	2.25
	3	1.10	3	0.37	3	0.52	3	2.02
	4	0.79	4	0.36	4	0.53	4	1.53
Average (mg/ml)	**2.05**		**0.69**		**0.85**		**2.61**	
Average (mg/g)	**93.09**		**31.54**		**38.73**		**118.45**	
Replicate 2	1	4.29	1	1.47	1	1.52	1	4.72
	2	1.66	2	0.60	2	0.70	2	2.24
	3	1.11	3	0.37	3	0.57	3	1.98
	4	0.80	4	0.33	4	0.66	4	1.57
Average (mg/ml)	**1.97**		**0.69**		**0.86**		**2.63**	
Average (mg/g)	**89.36**		**31.45**		**39.23**		**119.41**	
Replicate 3	1	4.69	1	1.49	1	1.65	1	5.12
	2	1.80	2	0.62	2	0.79	2	2.40
	3	1.18	3	0.40	3	0.57	3	2.04
	4	0.93	4	0.43	4	0.60	4	1.59
Average (mg/ml)	**2.15**		**0.74**		**0.90**		**2.79**	
Average (mg/g)	**97.73**		**33.45**		**41.04**		**126.64**	
Mean (mg/ml, n = 3)	**2.06**		**0.71**		**0.87**		**2.67**	
Mean (mg/g, n = 3)	**92.88**		**31.95**		**39.46**		**120.81**	

Appendix Table 5.3. Concentrations of Osborne protein fractions (albumin, globulin, prolamin, and glutelin) extracted from Native Millet across three biological replicates. Each replicate includes four technical extractions. Protein concentrations are expressed in mg/mL and converted to mg/g of sample. Reported values include individual extraction results, replicate means, and overall mean concentrations (mg/mL and mg/g) for each protein fraction.

	Albumin		**Globulin**		**prolamin**		**Glutelin**	
	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)
Replicate 1	1	4.01	1	0.48	1	1.94	1	1.36
	2	1.33	2	0.25	2	0.55	2	0.69
	3	0.69	3	0.16	3	0.21	3	0.65
	4	0.50	4	0.17	4	0.16	4	0.57
Average (mg/ml)	**1.63**		**0.27**		**0.71**		**0.82**	
Average (mg/g)	**75.13**		**12. 18**		**32.78**		**37.47**	
Replicate 2	1	3.62	1	0.53	1	1.67	1	1.19
	2	1.24	2	0.23	2	0.38	2	0.62
	3	0.70	3	0.20	3	0.27	3	0.52
	4	0.56	4	0.12	4	0.18	4	0.52
Average (mg/ml)	**1.53**		**0.27**		**0.63**		**0.71**	
Average (mg/g)	**70.39**		**12.41**		**28.74**		**32.78**	
Replicate 3	1	3.68	1	0.49	1	1.71	1	1.25
	2	1.34	2	0.27	2	0.46	2	0.68
	3	0.70	3	0.22	3	0.21	3	0.59
	4	0.58	4	0.12	4	0.17	4	0.54
Average (mg/ml)	**1.58**		**0.27**		**0.64**		**0.76**	
Average (mg/g)	**72.41**		**12.55**		**29.29**		**35.17**	
Mean (mg/ml, n = 3)	**1.58**		**0.27**		**0.66**		**0.76**	
Mean (mg/g, n = 3)	**72.23**		**12.30**		**30.08**		**34.93**	

Appendix Table 5.4. Concentrations of Osborne protein fractions (albumin, globulin, prolamin, and glutelin) extracted from Weeping Grass across three biological replicates. Each replicate includes four technical extractions. Protein concentrations are expressed in mg/mL and converted to mg/g of sample. Reported values include individual extraction results, replicate means, and overall mean concentrations (mg/mL and mg/g) for each protein fraction.

	Albumin		**Globulin**		**prolamin**		**Glutelin**	
	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)
Replicate 1	1	1.97	1	1.07	1	0.60	1	1.99
	2	0.63	2	0.60	2	0.17	2	1.10
	3	0.37	3	0.36	3	0.08	3	0.88
	4	0.25	4	0.28	4	0.10	4	0.85
Average (mg/ml)	**0.81**		**0.58**		**0.24**		**1.21**	
Average (mg/g)	**38.43**		**27.52**		**11.23**		**54.96**	
Replicate 2	1	1.76	1	1.02	1	0.61	1	2.00
	2	0.53	2	0.57	2	0.19	2	1.65
	3	0.29	3	0.33	3	0.06	3	1.56
	4	0.24	4	0.32	4	0.05	4	1.52
Average (mg/ml)	**0.71**		**0.56**		**0.23**		**1.68**	
Average (mg/g)	**33.62**		**26.62**		**10.95**		**76.67**	
Replicate 3	1	1.87	1	1.13	1	0.63	1	1.82
	2	0.60	2	0.59	2	0.19	2	1.10
	3	0.57	3	0.39	3	0.08	3	0.93
	4	0.31	4	0.25	4	0.06	4	0.82
Average (mg/ml)	**0.84**		**0.59**		**0.24**		**1.17**	
Average (mg/g)	**40.00**		**28.05**		**11.43**		**53.32**	
Mean (mg/ml, n = 3)	**0.78**		**0.58**		**0.24**		**1.35**	
Mean (mg/g, n = 3)	**37.11**		**27.22**		**11.12**		**61.66**	

Appendix Table 5.5. Concentrations of Osborne protein fractions (albumin, globulin, prolamin, and glutelin) extracted from barley across three biological replicates. Each replicate includes four technical extractions. Protein concentrations are expressed in mg/mL and converted to mg/g of sample. Reported values include individual extraction results, replicate means, and overall mean concentrations (mg/mL and mg/g) for each protein fraction.

	Albumin		**Globulin**		**prolamin**		**Glutelin**	
	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)	Extraction	Concentration (mg/ml)
Replicate 1	1	0.63	1	0.78	1	1.19	1	2.80
	2	0.52	2	0.19	2	0.64	2	0.47
	3	0.47	3	0.13	3	0.36	3	0.10
	4	0.51	4	0.15	4	0.06	4	0.11
Average (mg/ml)	**0.53**		**0.31**		**0.56**		**0.87**	
Average (mg/g)	**25.43**		**14.76**		**26.86**		**41.43**	
Replicate 2	1	0.84	1	0.72	1	1.11	1	2.35
	2	0.42	2	0.28	2	0.72	2	0.30
	3	0.34	3	0.06	3	0.36	3	0.11
	4	0.58	4	0.07	4	0.31	4	0.04
Average (mg/ml)	**0.55**		**0.28**		**0.63**		**0.70**	
Average (mg/g)	**26.19**		**13.43**		**29.76**		**33.38**	
Replicate 3	1	0.96	1	0.83	1	1.04	1	2.74
	2	0.58	2	0.29	2	0.69	2	0.89
	3	0.42	3	0.19	3	0.15	3	0.11
	4	0.32	4	0.06	4	0.06	4	0.05
Average (mg/ml)	**0.57**		**0.34**		**0.48**		**0.94**	
Average (mg/g)	**27.09**		**16.24**		**23.05**		**45.00**	
Mean (mg/ml, n = 3)	**0.55**		**0.31**		**0.56**		**0.84**	
Mean (mg/g, n = 3)	**26.10**		**14.76**		**26.46**		**39.79**	

Appendix Table 6.1. Top 100 most abundant proteins identified in Button Grass based on peptide-spectrum matches (PSMs), as determined by LC–MS/MS analysis and matched to the Swiss-Prot Green Plants database. Proteins are listed with their descriptions, matched species, and UniProt IDs.

Protein Description	**Matched Species (from Database)**	**UniProt ID**
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Oryza glaberrima*	Q42968
Late embryogenesis abundant protein, group 3	*Zea mays*	Q42376
Late embryogenesis abundant protein B19.1A	*Hordeum vulgare*	Q05190
Luminal-binding protein 3	*Zea mays*	O24581
50S ribosomal protein L2, chloroplastic	*Physcomitrium patens*	P60407
Heat shock 70 kDa protein 4	*Arabidopsis thaliana*	Q9LHA8
Fructose-bisphosphate aldolase, cytoplasmic isozyme	*Zea mays*	P08440
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 1	*Oryza sativa subsp. japonica*	Q2QLY5
Heat shock cognate 70 kDa protein 2	*Solanum lycopersicum*	P27322
ATP synthase subunit beta, mitochondrial	*Zea mays*	P19023
Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	*Oryza sativa* subsp. *japonica*	Q7FAH2
Chloroplast envelope membrane 70 kDa heat shock-related protein	*Spinacia oleracea*	P29357
Probable 1-Cys peroxiredoxin (Fragment)	*Bromus secalinus*	P52571
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Triticum aestivum*	P27736
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 2	*Oryza sativa* subsp. *japonica*	Q2QLY4
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Petunia hybrida*	P26520
Oleosin Zm-II	*Zea mays*	P21641
ATP synthase subunit beta-3, mitochondrial	*Arabidopsis thaliana*	Q9C5A9
Heat shock 70 kDa protein 2	*Arabidopsis thaliana*	P22954
Protein disulfide isomerase-like 1-1	*Oryza sativa* subsp. *japonica*	Q53LQ0
Peptidyl-prolyl cis-trans isomerase	*Zea mays*	P21569
Probable N6-adenosine-methyltransferase MT-A70-like	*Oryza sativa* subsp. *japonica*	Q6EU10
Protein SLE1	*Glycine max*	I1N2Z5
Phosphoglucomutase, cytoplasmic 1	*Zea mays*	P93804
Protein disulfide-isomerase	*Zea mays*	P52588
ADP, ATP carrier protein, mitochondrial	*Oryza sativa* subsp. *japonica*	P31691
Heat shock protein 81-1	*Oryza sativa* subsp. *indica*	A2YWQ1
Heat shock 70 kDa protein	*Chlamydomonas reinhardtii*	P25840
Protein disulfide-isomerase	*Hordeum vulgare*	P80284
ATP synthase subunit alpha, mitochondrial	*Triticum aestivum*	P12862
Probable pyridoxal 5'-phosphate synthase subunit PDX1.1	*Oryza sativa* subsp. *japonica*	Q69LA6
Putative aconitate hydratase, cytoplasmic	*Oryza sativa* subsp. *japonica*	Q6YZX6
Late embryogenesis abundant protein B19.1B	*Hordeum vulgare*	P46532
Actin-1	*Sorghum bicolor*	P53504
Stromal 70 kDa heat shock-related protein, chloroplastic	*Spinacia oleracea*	Q08080
Enolase OS=Oryza sativa subsp. japonica	*Oryza sativa* subsp. *japonica*	Q42971
Non-specific lipid-transfer protein	*Eleusine coracana*	P23802

5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	*Plectranthus scutellarioides*	Q42662
Peptidyl-prolyl cis-trans isomerase CYP20-1	*Arabidopsis thaliana*	Q9SP02
Soluble starch synthase 2-3, chloroplastic/amyloplastic	*Oryza sativa* subsp. *japonica*	Q0DDE3
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Ipomoea batatas*	Q42857
Alcohol dehydrogenase 1	*Oryza sativa* subsp. *indica*	Q75ZX4
Aldose reductase	*Hordeum vulgare*	P23901
Peptidyl-prolyl cis-trans isomerase	*Catharanthus roseus*	Q39613
Probable auxin efflux carrier component 9	*Oryza sativa* subsp. *japonica*	Q5VQY3
Elongation factor 1-alpha	*Oryza sativa* subsp. *japonica*	O64937
ADP, ATP carrier protein 1, mitochondrial	*Arabidopsis thaliana*	P31167
Malate dehydrogenase 2, mitochondrial	*Arabidopsis thaliana*	Q9LKA3
Inositol-3-phosphate synthase	*Hordeum vulgare*	O65195
Chaperonin CPN60-2, mitochondrial	*Zea mays*	Q43298
Formate dehydrogenase, mitochondrial	*Solanum tuberosum*	Q07511
Adenine nucleotide transporter BT1, chloroplastic/amyloplastic/mitochondrial	*Zea mays*	P29518
Germin-like protein subfamily 2 member 2	*Arabidopsis thaliana*	Q9FZ27
Triosephosphate isomerase, cytosolic	*Petunia hybrida*	P48495
Eukaryotic initiation factor 4A-3	*Oryza sativa* subsp. *japonica*	Q6Z2Z4
Spermidine synthase 1	*Oryza sativa* subsp. *japonica*	Q9SMB1
Ketol-acid reductoisomerase, chloroplastic	*Oryza sativa* subsp. *japonica*	Q65XK0
Cupincin	*Oryza sativa* subsp. *indica*	B8AL97
Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	*Oryza sativa* subsp. *japonica*	Q6K9N6
RuBisCO large subunit-binding protein subunit alpha, chloroplastic	*Triticum aestivum*	P08823
Embryonic protein DC-8	*Daucus carota*	P20075
RuBisCO large subunit-binding protein subunit beta, chloroplastic	*Pisum sativum*	P08927
Peptidyl-prolyl cis-trans isomerase	*Lupinus luteus*	O49886
Heat shock protein 82	*Zea mays*	Q08277
Chaperonin CPN60-1, mitochondrial	*Cucurbita maxima*	Q05045
Plasma membrane ATPase	*Oryza sativa* subsp. *japonica*	Q7XPY2
Cilia- and flagella-associated protein 65	*Chlamydomonas reinhardtii*	A8JFU2
Late embryogenesis abundant protein EMB564	*Zea mays*	P46517
Formate dehydrogenase, mitochondrial	*Hordeum vulgare*	Q9ZRI8
Xylose isomerase	*Hordeum vulgare*	Q40082
40S ribosomal protein S19	*Oryza sativa* subsp. *japonica*	P40978
Aspartate aminotransferase, cytoplasmic	*Oryza sativa* subsp. *japonica*	P37833
Endoplasmin homolog	*Hordeum vulgare*	P36183
Proteasome subunit alpha type-5	*Oryza sativa* subsp. *japonica*	Q9LSU1
Phosphoglycerate kinase, cytosolic	*Triticum aestivum*	P12783
Heat shock 70 kDa protein 7, chloroplastic	*Arabidopsis thaliana*	Q9LTX9
Cell division control protein 48 homolog E	*Arabidopsis thaliana*	Q9LZF6
16.9 kDa class I heat shock protein 1	*Oryza sativa* subsp. *japonica*	P27777

V-type proton ATPase catalytic subunit A	*Brassica napus*	Q39291
1,4-alpha-glucan-branching enzyme, chloroplastic/amyloplastic	*Oryza sativa* subsp. *japonica*	Q01401
Probable glutathione S-transferase DHAR1, cytosolic	*Oryza sativa* subsp. *japonica*	Q65XA0
REF/SRPP-like protein Os05g0151300/LOC_Os05g05940	*Oryza sativa* subsp. *japonica*	Q9FRA7
Malate dehydrogenase 2, peroxisomal	*Arabidopsis thaliana*	Q9ZP05
Probable alpha-glucosidase Os06g0675700	*Oryza sativa* subsp. *japonica*	Q653V7
Late embryogenesis abundant protein 1	*Oryza sativa* subsp. *indica*	A2XG55
Stromal 70 kDa heat shock-related protein, chloroplastic	*Pisum sativum*	Q02028
Neutral ceramidase	*Oryza sativa* subsp. *japonica*	Q0JL46
26S proteasome regulatory subunit 6A homolog	*Oryza sativa* subsp. *japonica*	P46465
Aldehyde dehydrogenase 1	*Tanacetum cinerariifolium*	A0A2I7G3B0
Proteasome subunit beta type-6	*Nicotiana tabacum*	P93395
Protein H2A.7	*Triticum aestivum*	Q43312
Chaperone protein ClpB1	*Oryza sativa* subsp. *japonica*	Q6F2Y7
Heat shock 70 kDa protein 10, mitochondrial	*Arabidopsis thaliana*	Q9LDZ0
D-3-phosphoglycerate dehydrogenase 3, chloroplastic	*Arabidopsis thaliana*	Q9LT69
RuBisCO large subunit-binding protein subunit alpha, chloroplastic	*Brassica napus*	P21239
Pyruvate decarboxylase 2	*Arabidopsis thaliana*	Q9FFT4
Glucose-1-phosphate adenylyltransferase small subunit 2, chloroplastic/amyloplastic/cytosolic	*Oryza sativa* subsp. *japonica*	P15280
17.0 kDa class II heat shock protein	*Zea mays*	Q08275
UTP--glucose-1-phosphate uridylyltransferase	*Hordeum vulgare*	Q43772
Chaperone protein ClpB1	*Arabidopsis thaliana*	P42730

Appendix Table 6.2. Top 100 most abundant proteins identified in Curly Mitchell Grass based on peptide-spectrum matches (PSMs), as determined by LC–MS/MS analysis and matched to the Swiss-Prot Green Plants database. Proteins are listed with their descriptions, matched species, and UniProt IDs.

Protein Description	**Matched Species (from Database)**	**UniProt ID**
Late embryogenesis abundant protein, group 3	*Triticum aestivum*	Q03968
Late embryogenesis abundant protein, group 3	*Zea mays*	Q42376
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Oryza sativa*	P0C585
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Sorghum bicolor*	Q43134
Luminal-binding protein 3	*Zea mays*	O24581
Heat shock 70 kDa protein 4	*Arabidopsis thaliana*	Q9LHA8
Heat shock 70 kDa protein BIP1	*Oryza sativa* subsp. *japonica*	Q6Z7B0
Heat shock 70 kDa protein BIP2	*Arabidopsis thaliana*	Q39043
Cruciferin CRU1	*Brassica napus*	P33525
Heat shock cognate 70 kDa protein 2	*Solanum lycopersicum*	P27322
Heat shock cognate 70 kDa protein	*Petunia hybrida*	P09189
Chloroplast envelope membrane 70 kDa heat shock-related protein	*Spinacia oleracea*	P29357
Heat shock 70 kDa protein 2	*Arabidopsis thaliana*	P22954
Fructose-bisphosphate aldolase, cytoplasmic isozyme	*Zea mays*	P08440
Actin	*Gossypium hirsutum*	O81221
Late embryogenesis abundant protein B19.1A	*Hordeum vulgare*	Q05190
ATP synthase subunit beta, mitochondrial	*Zea mays*	P19023
Chaperonin CPN60-2, mitochondrial	*Zea mays*	Q43298
50S ribosomal protein L2, chloroplastic	*Physcomitrium patens*	P60407
Non-specific lipid-transfer protein	*Eleusine coracana*	P23802
Elongation factor 1-alpha	*Nicotiana tabacum*	P43643
Phosphoglucomutase, cytoplasmic 2	*Zea mays*	P93805
Heat shock protein 81-1	*Oryza sativa* subsp. *indica*	A2YWQ1
Protein disulfide isomerase-like 1-1	*Oryza sativa* subsp. *japonica*	Q53LQ0
5-epiaristolochene 1,3-dihydroxylase	*Nicotiana tabacum*	Q94FM7
Cruciferin BnC2	*Brassica napus*	P33524
Protein disulfide-isomerase	*Zea mays*	P52588
Ketol-acid reductoisomerase, chloroplastic	*Oryza sativa* subsp. *japonica*	Q65XK0
ATP synthase subunit alpha, mitochondrial	*Triticum aestivum*	P12862
Late embryogenesis abundant protein EMB564	*Zea mays*	P46517
Actin-1	*Zea mays*	P02582
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Petunia hybrida*	P26520
Ketol-acid reductoisomerase, chloroplastic	*Spinacia oleracea*	Q01292
Heat shock protein 81-3	*Oryza sativa* subsp. *japonica*	Q07078
Enolase	*Oryza sativa* subsp. *japonica*	Q42971
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 1	*Oryza sativa* subsp. *japonica*	Q2QLY5
Ubiquitin-60S ribosomal protein L40-1	*Oryza sativa* subsp. *japonica*	P0CH34

Probable pyridoxal 5'-phosphate synthase subunit PDX1.1	*Oryza sativa* subsp. *japonica*	Q69LA6
Heat shock 70 kDa protein 6, chloroplastic	*Arabidopsis thaliana*	Q9STW6
ATP synthase subunit beta-3, mitochondrial	*Arabidopsis thaliana*	Q9C5A9
Chaperone protein ClpC1, chloroplastic	*Oryza sativa* subsp. *japonica*	Q7F9I1
Elongation factor 1-alpha	*Daucus carota*	P34823
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Pisum sativum*	P34922
Allergen Bra j 1-E	*Brassica juncea*	P80207
Chaperone protein ClpB2, chloroplastic	*Oryza sativa* subsp. *japonica*	Q75GT3
Oleosin Zm-II	*Zea mays*	P21641
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Pinus sylvestris*	P34924
1-Cys peroxiredoxin PER1	*Triticum aestivum*	Q6W8Q2
Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	*Hordeum vulgare*	P26517
Peptidyl-prolyl cis-trans isomerase	*Zea mays*	P21569
Stromal 70 kDa heat shock-related protein, chloroplastic	*Pisum sativum*	Q02028
Oleosin S2-2	*Brassica napus*	C3S7F1
Putative aconitate hydratase, cytoplasmic	*Oryza sativa* subsp. *japonica*	Q6YZX6
Chaperone protein ClpB1	*Oryza sativa* subsp. *japonica*	Q6F2Y7
Fructose-bisphosphate aldolase 2, cytoplasmic	*Oryza sativa* subsp. *japonica*	Q10A30
Heat shock protein 82	*Zea mays*	Q08277
Heat shock 70 kDa protein	*Chlamydomonas reinhardtii*	P25840
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	*Oryza sativa* subsp. *japonica*	Q6ZDY8
Cell division control protein 48 homolog E	*Arabidopsis thaliana*	Q9LZF6
Cell division cycle protein 48 homolog	*Glycine max*	P54774
Probable glutathione S-transferase DHAR1, cytosolic	*Oryza sativa* subsp. *japonica*	Q65XA0
Elongation factor 1-alpha	*Manihot esculenta*	O49169
Heat shock 70 kDa protein, mitochondrial	*Phaseolus vulgaris*	Q01899
Fructose-bisphosphate aldolase, cytoplasmic isozyme 2	*Pisum sativum*	P46257
Actin-71	*Solanum tuberosum*	P30168
Heat shock protein 90-1	*Arabidopsis thaliana*	P27323
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Triticum aestivum*	P27736
Chaperone protein ClpB3, mitochondrial	*Oryza sativa* subsp. *japonica*	Q0E3C8
Heat shock 70 kDa protein 10, mitochondrial	*Arabidopsis thaliana*	Q9LDZ0
Adenosylhomocysteinase	*Triticum aestivum*	P32112
Alcohol dehydrogenase 1	*Oryza sativa* subsp. *indica*	Q75ZX4
Tubulin beta-1 chain	*Eleusine indica*	Q9ZPP0
UTP--glucose-1-phosphate uridylyltransferase	*Hordeum vulgare*	Q43772
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Sinapis alba*	P04796
ATP synthase subunit alpha, mitochondrial	*Oenothera biennis*	P05492
Cruciferin CRU4	*Brassica napus*	P33522
Embryonic abundant protein 1	*Oryza sativa* subsp. *japonica*	P46520
Calmodulin-related protein	*Petunia hybrida*	P27164

Endoplasmin homolog	*Hordeum vulgare*	P36183
Phosphoglycerate kinase, cytosolic	*Triticum aestivum*	P12783
Protein disulfide-isomerase	*Hordeum vulgare*	P80284
Eukaryotic initiation factor 4A-3	*Oryza sativa* subsp. *japonica*	Q6Z2Z4
RuBisCO large subunit-binding protein subunit beta, chloroplastic	*Secale cereale*	Q43831
ADP, ATP carrier protein 2, mitochondrial	*Zea mays*	P12857
Cupincin	*Oryza sativa* subsp. *indica*	B8AL97
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Ipomoea batatas*	Q42857
RuBisCO large subunit-binding protein subunit alpha, chloroplastic	*Triticum aestivum*	P08823
Malate dehydrogenase, mitochondrial	*Citrullus lanatus*	P17783
Late embryogenesis abundant protein 76	*Brassica napus*	P13934
ATP-dependent Clp protease ATP-binding subunit ClpA homolog CD4B, chloroplastic	*Solanum lycopersicum*	P31542
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Craterostigma plantagineum*	Q42671
Malate dehydrogenase, mitochondrial	*Eucalyptus gunnii*	P46487
Heat shock 70 kDa protein, mitochondrial	*Pisum sativum*	P37900
Stromal 70 kDa heat shock-related protein, chloroplastic	*Spinacia oleracea*	Q08080
Elongation factor 1-alpha 1	*Arabidopsis thaliana*	P0DH99
UTP--glucose-1-phosphate uridylyltransferase	*Musa acuminata*	Q9SDX3
ATP synthase subunit alpha, mitochondrial	*Arabidopsis thaliana*	P92549
Tubulin alpha-1 chain	*Zea mays*	P14640
40S ribosomal protein S4	*Oryza sativa* subsp. *japonica*	P49398
40S ribosomal protein SA	*Glycine max*	O22518

Appendix Table 6.3. Top 100 most abundant proteins identified in Native Millet based on peptide-spectrum matches (PSMs), as determined by LC–MS/MS analysis and matched to the Swiss-Prot Green Plants database. Proteins are listed with their descriptions, matched species, and UniProt IDs.

Protein Description	**Matched Species (from Database)**	**UniProt ID**
ABA-inducible protein PHV A1	*Hordeum vulgare*	P14928
Late embryogenesis abundant protein, group 3	*Zea mays*	Q42376
Granule-bound starch synthase 1, chloroplastic/amyloplastic PE=3 SV=1	*Zea mays*	P04713
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Oryza glaberrima*	Q42968
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Triticum aestivum*	P27736
50S ribosomal protein L2, chloroplastic	*Physcomitrium patens*	P60407
Late embryogenesis abundant protein EMB564	*Zea mays*	P46517
Chaperonin CPN60-2, mitochondrial	*Zea mays*	Q43298
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Antirrhinum majus*	P25861
Oleosin 18 kDa	*Oryza sativa* subsp. *indica*	A2XL05
Luminal-binding protein 3	*Zea mays*	O24581
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Magnolia liliiflora*	P26518
Chloroplast envelope membrane 70 kDa heat shock-related protein	*Spinacia oleracea*	P29357
RuBisCO large subunit-binding protein subunit beta, chloroplastic	*Secale cereale*	Q43831
63 kDa globulin-like protein	*Oryza sativa* subsp. *japonica*	Q75GX9
Phosphoglucomutase, cytoplasmic 1	*Zea mays*	P93804
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 1	*Oryza sativa* subsp. *japonica*	Q2QLY5
Heat shock 70 kDa protein 4	*Arabidopsis thaliana*	Q9LHA8
5-epiaristolochene 1,3-dihydroxylase	*Nicotiana tabacum*	Q94FM7
Heat shock 70 kDa protein 6, chloroplastic	*Arabidopsis thaliana*	Q9STW6
Heat shock 70 kDa protein 18	*Arabidopsis thaliana*	Q9C7X7
Heat shock 70 kDa protein	*Chlamydomonas reinhardtii*	P25840
Formate dehydrogenase 1, mitochondrial	*Oryza sativa subsp. japonica*	Q9SXP2
Late embryogenesis abundant protein B19.1A	*Hordeum vulgare*	Q05190
Peptidyl-prolyl cis-trans isomerase	*Zea mays*	P21569
ATP synthase subunit alpha, mitochondrial	*Triticum aestivum*	P12862
Sucrose synthase 1	*Hordeum vulgare*	P31922
Heat shock cognate 70 kDa protein	*Petunia hybrida*	P09189
Non-specific lipid-transfer protein 2	*Sorghum bicolor*	Q43194
Malate dehydrogenase, mitochondrial	*Eucalyptus gunnii*	P46487
Stromal 70 kDa heat shock-related protein, chloroplastic	*Spinacia oleracea*	Q08080
Ubiquitin-60S ribosomal protein L40-1	*Oryza sativa* subsp. *japonica*	P0CH34
ATP synthase subunit beta, mitochondrial	*Zea mays*	P19023
Probable pyridoxal 5'-phosphate synthase subunit PDX1.1	*Oryza sativa* subsp. *japonica*	Q69LA6
Alcohol dehydrogenase 1	*Zea mays*	P00333
RuBisCO large subunit-binding protein subunit alpha, chloroplastic	*Triticum aestivum*	P08823
Chaperonin 60 subunit beta 1, chloroplastic	*Arabidopsis thaliana*	P21240

RuBisCO large subunit-binding protein subunit alpha,	*Brassica napus*	P21239
Protein MEI2-like 5	*Oryza sativa* subsp. *japonica*	Q6EQX3
40S ribosomal protein S19	*Oryza sativa* subsp. *japonica*	P40978
Elongation factor 1-alpha	*Zea mays*	Q41803
40S ribosomal protein S5 (Fragment)	*Nicotiana plumbaginifolia*	O24111
ADP,ATP carrier protein, mitochondrial	*Oryza sativa* subsp. *japonica*	P31691
Histone H2B.10	*Oryza sativa* subsp. *indica*	A2WKS3
14-3-3-like protein GF14-12	*Zea mays*	Q01526
Malate synthase, glyoxysomal	*Zea mays*	P49081
Hsp70-Hsp90 organizing protein	*Triticum aestivum*	F8RP11
Adenosylhomocysteinase	*Phalaenopsis sp.*	P50249
Actin-71	*Solanum tuberosum*	P30168
ATP-citrate synthase beta chain protein 1	*Oryza sativa* subsp. *japonica*	Q93VT8
Putative aconitate hydratase, cytoplasmic	*Oryza sativa* subsp. *japonica*	Q6YZX6
Nucleoside diphosphate kinase 1	*Mesembryanthemum crystallinum*	O81372
Late embryogenesis abundant protein B19.3	*Hordeum vulgare*	Q02400
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 2	*Arabidopsis thaliana*	Q9SRV5
Protein disulfide-isomerase	*Zea mays*	P52588
Eukaryotic translation initiation factor 5A	*Zea mays*	P80639
Actin-104 (Fragment)	*Nicotiana tabacum*	P93375
Inositol-3-phosphate synthase	*Hordeum vulgare*	O65195
Tubulin beta-1 chain	*Oryza sativa* subsp. *japonica*	Q43594
Aspartate aminotransferase, cytoplasmic	*Oryza sativa* subsp. *japonica*	P37833
Leucine aminopeptidase 2, chloroplastic	*Oryza sativa* subsp. *japonica*	Q6K669
Tubulin beta chain (Fragment)	*Glycine max*	P28551
Phosphoglycerate kinase, cytosolic	*Triticum aestivum*	P12783
16.9 kDa class I heat shock protein 1	*Oryza sativa* subsp. *japonica*	P27777
V-type proton ATPase catalytic subunit A	*Brassica napus*	Q39291
Nucleosome assembly protein 1;4	*Nicotiana tabacum*	Q70Z16
Catalase isozyme A	*Oryza sativa* subsp. *indica*	P0C549
Probable calcium-binding protein CML7	*Oryza sativa* subsp. *japonica*	Q84VG0
ATP synthase subunit alpha, chloroplastic	*Oryza nivara*	Q6ENH7
14-3-3-like protein A	*Hordeum vulgare*	P29305
Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial	*Oryza sativa* subsp. *japonica*	Q6K9N6
60S ribosomal protein L12	*Prunus armeniaca*	O50003
Glucose and ribitol dehydrogenase homolog	*Oryza sativa* subsp. *japonica*	Q75KH3
Chaperone protein ClpB2, chloroplastic	*Oryza sativa* subsp. *japonica*	Q75GT3
Ricin B-like lectin R40C1	*Oryza sativa* subsp. *japonica*	Q10M12
Heat shock 70 kDa protein 10, mitochondrial	*Arabidopsis thaliana*	Q9LDZ0
Thiamine thiazole synthase 1, chloroplastic	*Zea mays*	Q41738
Glycine-rich RNA-binding protein GRP1A	*Sinapis alba*	P49310

6-phosphogluconate dehydrogenase, decarboxylating 2, chloroplastic	*Oryza sativa* subsp. *japonica*	Q2R480
Pyruvate, phosphate dikinase 1, chloroplastic	*Arabidopsis thaliana*	O23404
Protein SLE1	*Glycine max*	I1N2Z5
Probable mitochondrial-processing peptidase subunit alpha-1, mitochondrial	*Arabidopsis thaliana*	Q9ZU25
Nucleoside diphosphate kinase 1	*Zea mays*	B4FK49
1,4-alpha-glucan-branching enzyme 2, chloroplastic/amyloplastic	*Zea mays*	Q08047
Superoxide dismutase [Mn] 3.4, mitochondrial	*Zea mays*	P41980
UTP--glucose-1-phosphate uridylyltransferase	*Hordeum vulgare*	Q43772
Chaperone protein ClpB1	*Arabidopsis thaliana*	P42730
Equilibrative nucleotide transporter 7	*Arabidopsis thaliana*	Q944P0
2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*Zea mays*	P30792
Fructose-bisphosphate aldolase, cytoplasmic isozyme	*Zea mays*	P08440
Elongation factor 1-gamma 2	*Oryza sativa* subsp. *japonica*	Q6YW46
Pyruvate dehydrogenase E1 component subunit alpha-3, chloroplastic	*Oryza sativa* subsp. *japonica*	Q7XTJ3
ATP synthase subunit alpha, mitochondrial	*Oenothera biennis*	P05492
Embryonic abundant protein 1	*Oryza sativa* subsp. *japonica*	P46520
Enolase	*Oryza sativa* subsp. *japonica*	Q42971
Proteasome subunit alpha type-2	*Oryza sativa* subsp. *indica*	A2YVR7
19.0 kDa class II heat shock protein	*Oryza sativa* subsp. *japonica*	Q6Z6L5
ATP synthase subunit alpha, chloroplastic	*Helianthus annuus*	Q1KXW5
Probable histone H2A.1	*Oryza sativa* subsp. *indica*	A2YMC5
Probable ascorbate-specific transmembrane electron transporter 2	*Oryza sativa* subsp. *japonica*	Q7XMK3

Appendix Table 6.4. Top 100 most abundant proteins identified in Weeping Grass based on peptide-spectrum matches (PSMs), as determined by LC–MS/MS analysis and matched to the Swiss-Prot Green Plants database. Proteins are listed with their descriptions, matched species, and UniProt IDs.

Protein Description	**Matched Species (from Database)**	**UniProt ID**
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Zea mays*	P04713
Divinyl chlorophyllide a 8-vinyl-reductase, chloroplastic	*Oryza sativa* subsp. *indica*	D5L1S4
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Oryza glaberrima*	Q42968
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Triticum aestivum*	P27736
Glutelin type-B 1	*Oryza sativa* subsp. *japonica*	P14323
Glutelin type-A 2	*Oryza sativa* subsp. *japonica*	P07730
Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	*Oryza sativa* subsp. *japonica*	Q7FAH2
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Antirrhinum majus*	P25861
Glutelin type-A 3	*Oryza sativa* subsp. *japonica*	Q09151
Heat shock 70 kDa protein 2	*Arabidopsis thaliana*	P22954
Heat shock 70 kDa protein	*Zea mays*	P11143
12S seed storage globulin 1	*Avena sativa*	P12615
Ketol-acid reductoisomerase, chloroplastic	*Oryza sativa* subsp. *japonica*	Q65XK0
RuBisCO large subunit-binding protein subunit beta, chloroplastic	*Secale cereale*	Q43831
1-Cys peroxiredoxin A	*Oryza sativa* subsp. *japonica*	P0C5C9
26.7 kDa heat shock protein, chloroplastic	*Oryza sativa* subsp. *japonica*	Q10P60
Chaperone protein ClpB2, chloroplastic	*Oryza sativa* subsp. *japonica*	Q75GT3
Pyruvate, phosphate dikinase 1, chloroplastic	*Zea mays*	P11155
ATP synthase subunit alpha, mitochondrial	*Oryza sativa*	P0C520
Actin-104 (Fragment)	*Nicotiana tabacum*	P93375
16.9 kDa class I heat shock protein 1	*Oryza sativa* subsp. *japonica*	P27777
ATP synthase subunit beta, mitochondrial	*Zea mays*	P19023
Pyruvate, phosphate dikinase 2	*Zea mays*	Q42368
Lactoylglutathione lyase	*Oryza sativa* subsp. *japonica*	Q948T6
Luminal-binding protein 5	*Nicotiana tabacum*	Q03685
16.9 kDa class I heat shock protein 2	*Oryza sativa* subsp. *japonica*	Q943E6
Proteasome subunit alpha type-5	*Oryza sativa* subsp. *japonica*	Q9LSU1
1,4-alpha-glucan-branching enzyme, chloroplastic/amyloplastic	*Oryza sativa* subsp. *japonica*	Q01401
Late embryogenesis abundant protein 1	*Oryza sativa* subsp. *indica*	A2XG55
Probable phosphoglucomutase, cytoplasmic 2	*Arabidopsis thaliana*	Q9SGC1
Endoplasmic reticulum chaperone BiP	*Corylus avellana*	Q9FSY7
Embryonic protein DC-8	*Daucus carota*	P20075
Carrot ABA-induced in somatic embryos 3	*Daucus carota*	Q5KTS7
Oleosin Zm-II	*Zea mays*	P21641
RuBisCO large subunit-binding protein subunit alpha, chloroplastic	*Brassica napus*	P21239
Glutaredoxin-C6	*Oryza sativa* subsp. *japonica*	P55142
ATP synthase subunit beta, mitochondrial	*Chlamydomonas reinhardtii*	P38482

Enolase 2	*Zea mays*	P42895
Phosphoenolpyruvate carboxylase 2	*Sorghum bicolor*	P29194
Alpha-(1,4)-fucosyltransferase	*Arabidopsis thaliana*	Q9C8W3
Chaperonin CPN60-2, mitochondrial	*Zea mays*	Q43298
Squamosa promoter-binding-like protein 7	*Arabidopsis thaliana*	Q8S9G8
Elongation factor 1-alpha	*Hordeum vulgare*	P34824
Pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial	*Oryza sativa* subsp. *japonica*	Q6Z1G7
GTP-binding nuclear protein Ran-1	*Arabidopsis thaliana*	P41916
Protein disulfide isomerase-like 1-1	*Oryza sativa* subsp. *japonica*	Q53LQ0
Histone H2B.3 (Fragment)	*Solanum lycopersicum*	O65819
Chaperonin 60 subunit beta 3, chloroplastic	*Arabidopsis thaliana*	C0Z361
Nucleoside diphosphate kinase 1	*Pisum sativum*	P47922
Proteasome subunit alpha type-7	*Solanum lycopersicum*	O24030
Peptidyl-prolyl cis-trans isomerase	*Zea mays*	P21569
40S ribosomal protein S19-2	*Arabidopsis thaliana*	Q9LF30
Soluble starch synthase 2-3, chloroplastic/amyloplastic	*Oryza sativa* subsp. *japonica*	Q0DDE3
Adenosylhomocysteinase	*Phalaenopsis sp.*	P50249
Enolase 1, chloroplastic	*Arabidopsis thaliana*	Q9C9C4
Probable pyridoxal 5'-phosphate synthase subunit PDX1.	*Oryza sativa* subsp. *japonica*	Q69LA6
Stromal 70 kDa heat shock-related protein, chloroplastic	*Pisum sativum*	Q02028
1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase homolog 2	*Sorghum bicolor*	C5WWY0
Triosephosphate isomerase, cytosolic	*Secale cereale*	P46226
RuBisCO large subunit-binding protein subunit alpha, chloroplastic	*Triticum aestivum*	P08823
Dehydrin DHN1	*Zea mays*	P12950
40S ribosomal protein S28	*Zea mays*	P46302
Fructose-bisphosphate aldolase 2, cytoplasmic	*Oryza sativa* subsp. *japonica*	Q10A30
40S ribosomal protein S10-1	*Oryza sativa* subsp. *japonica*	P0DKK8
Proliferating cell nuclear antigen	*Oryza sativa* subsp. *japonica*	P17070
Vicilin C72	*Gossypium hirsutum*	P09801
Disease resistance protein RUN1	*Vitis rotundifolia*	V9M398
Superoxide dismutase [Cu-Zn] 2	*Oryza sativa* subsp. *japonica*	P28757
17.0 kDa class II heat shock protein	*Zea mays*	Q08275
Malate dehydrogenase 2, cytoplasmic	*Arabidopsis thaliana*	P57106
Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase, chloroplastic	*Gossypium hirsutum*	Q6SJV8
Mitochondrial outer membrane protein porin 3	*Oryza sativa* subsp. *japonica*	Q7F4F8
40S ribosomal protein S20	*Oryza sativa* subsp. *japonica*	P35686
Sucrose synthase 2	*Hordeum vulgare*	P31923
Non-specific lipid-transfer protein	*Eleusine coracana*	P23802
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	*Plectranthus scutellarioides*	Q42662
Antifungal protein R	*Hordeum vulgare*	P33044
Aquaporin PIP1-5	*Zea mays*	Q9AR14

Late embryogenesis abundant protein 19	*Oryza sativa* subsp. *indica*	A2Y720
Tubulin beta-1 chain	*Oryza sativa* subsp. *japonica*	Q43594
Photosystem I P700 chlorophyll a apoprotein A2	*Helianthus annuus*	Q1KXW0
Aspartate aminotransferase, cytoplasmic	*Oryza sativa* subsp. *japonica*	P37833
40S ribosomal protein S4	*Oryza sativa* subsp. *japonica*	P49398
Endochitinase 3	*Nicotiana tabacum*	P29059
Eukaryotic initiation factor 4A	*Zea mays*	Q41741
Ras-related protein RABA5d	*Arabidopsis thaliana*	Q9SIP0
Leucine aminopeptidase 2, chloroplastic	*Oryza sativa* subsp. *japonica*	Q6K669
60S ribosomal protein L8-1	*Arabidopsis thaliana*	P46286
60S ribosomal protein L31	*Panax ginseng*	Q9MAV7
ATP-citrate synthase beta chain protein 1	*Arabidopsis thaliana*	Q9C522
Histone H3.3	*Hordeum vulgare*	P06353
Glucose-1-phosphate adenylyltransferase large subunit 2, cytosolic	*Oryza sativa* subsp. *japonica*	Q7G065
2-Cys peroxiredoxin BAS1, chloroplastic	*Oryza sativa* subsp. *japonica*	Q6ER94
Formate dehydrogenase, mitochondrial	*Solanum tuberosum*	Q07511
26S proteasome regulatory subunit 6A homolog A	*Arabidopsis thaliana*	Q9SEI2
40S ribosomal protein S27-2	*Arabidopsis thaliana*	Q9M2F1
23.2 kDa heat shock protein	*Oryza sativa* subsp. *japonica*	Q7XUW5
Heat shock protein 81-1	*Oryza sativa* subsp. *indica*	A2YWQ1
Ketol-acid reductoisomerase, chloroplastic	*Populus euphratica*	P84534
Betaine aldehyde dehydrogenase 2	*Oryza sativa* subsp. *indica*	B3VMC0

Appendix Table 6.5. Top 100 most abundant proteins identified in barley based on peptide-spectrum matches (PSMs), as determined by LC–MS/MS analysis and matched to the Swiss-Prot Green Plants database. Proteins are listed with their descriptions, matched species, and UniProt IDs.

Protein Description	**Matched Species (from Database)**	**UniProt ID**
ABA-inducible protein PHV A1	*Hordeum vulgare*	P14928
Late embryogenesis abundant protein, group 3	*Triticum aestivum*	Q03968
Late embryogenesis abundant protein B19.1A	*Hordeum vulgare*	Q05190
Protein disulfide-isomerase	*Hordeum vulgare*	P80284
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Hordeum vulgare*	P09842
Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	*Hordeum vulgare*	P08477
Prunin 1 Pru du 6.0101	*Prunus dulcis*	E3SH28
Aldose reductase	*Hordeum vulgare*	P23901
Em protein CS41	*Triticum aestivum*	P22701
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Zea mays*	P04713
Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	*Hordeum vulgare*	P26517
Protein disulfide-isomerase	*Triticum aestivum*	P52589
Histone H2B.10	*Oryza sativa subsp. indica*	A2WKS3
Beta-amylase OS=OX=77009 GN=BMY1 PE=1 SV=1	*Hordeum vulgare* subsp. *spontaneum*	P82993
Heat shock 70 kDa protein 4	*Arabidopsis thaliana*	Q9LHA8
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	*Antirrhinum majus*	P25861
B3-hordein	*Hordeum vulgare*	P06471
Heat shock cognate 70 kDa protein 2	*Solanum lycopersicum*	P27322
Heat shock cognate 70 kDa protein	*Petunia hybrida*	P09189
Late embryogenesis abundant protein B19.4	*Hordeum vulgare*	Q05191
1-Cys peroxiredoxin PER1	*Hordeum vulgare*	P52572
Late embryogenesis abundant protein B19.3	*Hordeum vulgare*	Q02400
Heat shock 70 kDa protein BIP1	*Oryza sativa* subsp. *japonica*	Q6Z7B0
Granule-bound starch synthase 1, chloroplastic/amyloplastic	*Oryza glaberrima*	Q42968
Heat shock cognate 70 kDa protein 1	*Solanum lycopersicum*	P24629
Enolase	*Oryza sativa* subsp. *japonica*	Q42971
16.9 kDa class I heat shock protein 2	*Triticum aestivum*	Q41560
Sucrose synthase 1	*Hordeum vulgare*	P31922
Polyubiquitin	*Acetabularia peniculus*	P42739
Heat shock 70 kDa protein BIP2	*Arabidopsis thaliana*	Q39043
Heat shock 70 kDa protein	*Glycine max*	P26413
ADP, ATP carrier protein, mitochondrial	*Oryza sativa* subsp. *japonica*	P31691
Heat shock 70 kDa protein 18	*Arabidopsis thaliana*	Q9C7X7
Endoplasmic reticulum chaperone BiP	*Corylus avellana*	Q9FSY7
Actin	*Gossypium hirsutum*	O81221
Serpin-Z7	*Hordeum vulgare*	Q43492
Chaperone protein ClpB1	*Oryza sativa* subsp. *japonica*	Q6F2Y7

17.0 kDa class II heat shock protein	*Zea mays*	Q08275
ADP,ATP carrier protein 1, mitochondrial	*Arabidopsis thaliana*	P31167
Luminal-binding protein	*Spinacia oleracea*	Q42434
Elongation factor 1-alpha	*Triticum aestivum*	Q03033
Phosphoglycerate kinase, cytosolic	*Triticum aestivum*	P12783
Peptidyl-prolyl cis-trans isomerase	*Zea mays*	P21569
ATP synthase subunit alpha, mitochondrial	*Triticum aestivum*	P12862
Probable pyridoxal 5'-phosphate synthase subunit PDX1.1	*Oryza sativa* subsp. *japonica*	Q69LA6
Linoleate 9S-lipoxygenase 1	*Hordeum vulgare*	P29114
Probable pyridoxal 5'-phosphate synthase subunit PDX1.2	*Oryza sativa* subsp. *japonica*	Q8W3D0
Glucose-1-phosphate adenylyltransferase large subunit, chloroplastic/amyloplastic	*Triticum aestivum*	P12299
Serpin-Z4	*Hordeum vulgare*	P06293
Protein synthesis inhibitor I	*Hordeum vulgare*	P22244
ATP synthase subunit beta, mitochondrial	*Zea mays*	P19023
RuBisCO large subunit-binding protein subunit beta, chloroplastic	*Secale cereale*	Q43831
Heat shock 70 kDa protein 10, mitochondrial	*Arabidopsis thaliana*	Q9LDZ0
Pyridoxal 5'-phosphate synthase subunit PDX1.1	*Arabidopsis thaliana*	O80448
Heat shock protein 82	*Zea mays*	Q08277
UTP--glucose-1-phosphate uridylyltransferase	*Hordeum vulgare*	Q43772
Heat shock protein 81-3	*Oryza sativa* subsp. *japonica*	Q07078
Fructose-bisphosphate aldolase, cytoplasmic isozyme	*Zea mays*	P08440
Sucrose synthase 2	*Hordeum vulgare*	P31923
Chaperonin CPN60-2, mitochondrial	*Zea mays*	Q43298
Protein synthesis inhibitor II	*Hordeum vulgare*	P04399
RuBisCO large subunit-binding protein subunit alpha, chloroplastic	*Triticum aestivum*	P08823
Phosphoglucomutase, cytoplasmic	*Bromus inermis*	Q9SNX2
Adenosylhomocysteinase	*Triticum aestivum*	P32112
Glucose-1-phosphate adenylyltransferase small subunit, chloroplastic/amyloplastic	*Hordeum vulgare*	P55238
Malate dehydrogenase, cytoplasmic	*Zea mays*	Q08062
Heat shock 70 kDa protein, mitochondrial OS=OX=3888 GN=HSP1 PE=2 SV=1	*Pisum sativum*	P37900
Heat shock protein 83	*Ipomoea nil*	P51819
Glycine-rich RNA-binding protein blt801	*Hordeum vulgare*	Q43472
26S proteasome regulatory subunit 6A homolog A	*Arabidopsis thaliana*	Q9SEI2
Heat shock protein 81-1	*Oryza sativa subsp. indica*	A2YWQ1
14-3-3-like protein A	*Hordeum vulgare*	P29305
Chaperone protein ClpB3, mitochondrial	*Oryza sativa* subsp. *japonica*	Q0E3C8
Pyruvate, phosphate dikinase 1, chloroplastic	*Arabidopsis thaliana*	O23404
Malate dehydrogenase, cytoplasmic	*Oryza sativa* subsp. *japonica*	Q7XDC8
Vicilin C72	*Gossypium hirsutum*	P09801
Triosephosphate isomerase, cytosolic	*Hordeum vulgare*	P34937
Chaperone protein ClpB1	*Arabidopsis thaliana*	P42730

40S ribosomal protein Sa-2	*Arabidopsis thaliana*	Q8H173
ATP synthase subunit alpha, mitochondrial	*Oenothera biennis*	P05492
RuBisCO large subunit-binding protein subunit alpha, chloroplastic	*Pisum sativum*	P08926
Endoplasmin homolog	*Hordeum vulgare*	P36183
Actin-1	*Zea mays*	P02582
Pyruvate, phosphate dikinase, chloroplastic	*Flaveria trinervia*	P22221
Heat shock 70 kDa protein 7, chloroplastic	*Arabidopsis thaliana*	Q9LTX9
Pyruvate, phosphate dikinase 2	*Oryza sativa* subsp. *japonica*	Q75KR1
Histone H4.3	*Zea mays*	Q41811
Lactoylglutathione lyase	*Oryza sativa* subsp. *japonica*	Q948T6
Clathrin heavy chain 1	*Oryza sativa* subsp. *japonica*	Q2RBN7
63 kDa globulin-like protein	*Oryza sativa* subsp. *japonica*	Q75GX9
Pyruvate kinase 2, cytosolic	*Oryza sativa* subsp. *indica*	B8BM17
14-3-3-like protein B	*Hordeum vulgare*	Q43470
Fructose-bisphosphate aldolase 2, cytoplasmic	*Oryza sativa* subsp. *japonica*	Q10A30
Chaperone protein ClpB2, chloroplastic	*Oryza sativa* subsp. *japonica*	Q75GT3
Catalase isozyme 1	*Hordeum vulgare*	P55307
Tubulin beta-3 chain	*Triticum aestivum*	Q9ZRB0
Pyruvate decarboxylase 3	*Oryza sativa* subsp. *japonica*	Q0D3D2
RuBisCO large subunit-binding protein subunit beta, chloroplastic	*Pisum sativum*	P08927
Pyridoxal 5'-phosphate synthase subunit PDX1	*Phaseolus vulgaris*	Q9FT25
Pyruvate decarboxylase 2	*Oryza sativa* subsp. *japonica*	Q10MW3