



University of Tuscia
Department of Agriculture and Forest Sciences (DAFNE)

PhD Programme in

Plant and Animal Science - XXXV Cycle

**"Exploring variability in free asparagine content to reduce
the acrylamide-forming potential of wheat grain"**

s.s.d. AGR/07

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Academic Year 2022/2023

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Abstract

Acrylamide, an odourless, organic and water-soluble molecule, has been detected as a contaminant in a wide range of foods that have been exposed to high temperatures during processing (e.g., fried, baked, roasted). Since acrylamide is classified as a probable human carcinogen, many efforts have been made to decrease its content in foods.

Acrylamide can be generated during Maillard cascade reactions involving free reducing sugars and free asparagine, the latter having been identified as the limiting factor in acrylamide formation in wheat containing foods. This makes the control of free asparagine content of great importance in crop and food sciences.

The aim of this PhD project was to study natural variation in free asparagine levels in durum (*Triticum turgidum* L. ssp. *durum* (Desf.)) and bread (*Triticum aestivum* L.) wheat grains, and to identify genomic regions involved in free asparagine accumulation in wheat seeds.

To achieve these goals, three research activities were carried out.

Within a first activity, genetic diversity in relation to free asparagine content in durum wheat was explored. For this purpose, 201 durum wheat genotypes, originating from 33 different countries, were selected from an international collection (the Global Durum Panel). To increase genetic variability, a large number of durum wheat landraces were included in the study. The 201 genotypes were sown in experimental fields located in Fiorenzuola d'Arda (Italy) for three years. Wholemeal flour was obtained from harvested grains and free asparagine was measured. The analysis highlighted the presence of significant variability for this trait in the chosen population. A multi-locus genome-wide association study allowed to identify genomic regions associated to free asparagine content on chromosomes 6A, 7A, 2B, 4B, 7B.

A second study aimed to characterise 54 bread wheat elite varieties, relevant for the Italian market, in order to identify genotypes with contrasting free asparagine accumulation levels and evaluate their acrylamide-forming during food processing. Wholemeal flour deriving from seeds harvested in six field trials

(three Italian locations, two years) were analysed for free asparagine content. Analysing the 18 genotypes that were present in all the field trials, we monitored how the trait was influenced by genotype and cultivation environment. Some cultivars appeared to be highly affected by environment, whereas others showed a relative stability in free asparagine content across years and locations. Among them, four genotypes were selected for their contrasting levels of free asparagine accumulation (2 genotypes showing low and 2 genotypes showing high free asparagine accumulation across years and cultivation environments). Acrylamide content was measured in pizza produced with flours obtained from these genotypes.

The third activity concerned the development of a gene editing protocol to modulate free asparagine content in durum wheat seeds. This work was carried out in collaboration with the research group of Prof. Nigel G. Halford in Rothamsted Research, (Harpenden, Hertfordshire, UK). During a period spent at Rothamsted Research, three guide RNAs (gRNAs) for CRISPR/Cas9-based editing were designed to target the first exon of *TdASN2* gene, a key gene in asparagine synthesis which is expressed at late stages of seed development. After the assembly of the vectors, particle bombardment technology was applied to deliver DNA into immature embryos. More than 700 immature embryos were bombarded and 10 plants regenerated from callus, two of which were found to be actually transformed. The activity is still ongoing, and the results obtained so far have evidenced the need to optimise selection media for regeneration and transformant selection.

Riassunto (in Italian)

L'acrilamide è una molecola organica, inodore e idrosolubile, che è stata rilevata come contaminante in un'ampia gamma di alimenti che sono esposti a temperature elevate durante la loro preparazione (ad es. sottoposti a cotture quali frittura, cottura al forno, cibi arrostiti). Poiché l'acrilamide è stata classificata come probabile cancerogeno per l'uomo, sono stati fatti molti sforzi per ridurre il contenuto negli alimenti.

L'acrilamide può essere generata durante la reazione di Maillard che coinvolge zuccheri riducenti e asparagina libera (non proteica). Quest'ultima è stata identificata come il fattore determinante nella formazione di acrilamide negli alimenti derivati dal frumento, rendendo il controllo del contenuto di asparagina libera di grande importanza in abito agrario e alimentare.

In questo progetto di dottorato è stata studiata la variabilità dei livelli di asparagina libera nella granella di frumento duro (*Triticum turgidum* L. ssp. *durum* (Desf.)) e frumento tenero (*Triticum aestivum* L.), e sono state identificate regioni genomiche associate all'accumulo di asparagina libera nei semi di questi due cereali.

Sono state svolte tre diverse attività di ricerca funzionali al raggiungimento degli obiettivi del progetto.

La prima attività mirava a esplorare la diversità genetica in relazione al contenuto di asparagina libera nel grano duro. A tale scopo sono stati selezionati, da una collezione internazionale (Global Durum Panel), 201 genotipi di grano duro, originari di 33 paesi diversi. Per garantire un'elevata variabilità genetica, nello studio è stato incluso un gran numero di *landraces*. I 201 genotipi sono stati seminati in campi sperimentali situati a Fiorenzuola d'Arda (Italia) per tre anni. Sono stati poi misurati i livelli di asparagina libera nella farina integrale ottenuta dalle cariossidi. L'analisi ha mostrato la presenza di una variabilità significativa per questo carattere nella popolazione selezionata. È stato quindi eseguito uno studio di associazione (multi-locus GWAS) per identificare le regioni genomiche associate al contenuto di

asparagina libera. Sono stati identificati polimorfismi a singolo nucleotide (SNP) sui cromosomi 6A, 7A, 2B, 4B, 7B associati al contenuto di asparagina libera.

Un secondo studio mirava a caratterizzare 54 varietà élite di frumento tenero di rilevanza per il mercato italiano con l'obiettivo di identificare genotipi con livelli di accumulo di asparagina libera differenziati e valutare la formazione di acrilamide durante la lavorazione degli alimenti prodotti a partire dalle loro farine. Le farine integrali derivanti dalla granella raccolta in sei prove in campo (tre località italiane, due anni) sono state analizzate per il contenuto di asparagina libera. Considerando i 18 genotipi presenti in tutte le prove di campo, è stato evidenziato come il carattere fosse influenzato dal genotipo e dall'ambiente di coltivazione. Per alcune cultivar i livelli sono risultati molto variabili a seconda dell'ambiente, mentre per altre si è osservata una relativa stabilità nel contenuto di asparagina libera nei diversi anni e nelle differenti località. Tra queste ultime, sono stati selezionati quattro genotipi con livelli differenziati di asparagina libera (2 genotipi che mostrano basso accumulo e 2 genotipi che mostrano alto accumulo di asparagina libera in diversi anni e ambienti di coltivazione). Successivamente è stato misurato il contenuto di acrilamide in pizze prodotte a partire da farine ottenute da questi genotipi.

La terza attività ha riguardato lo sviluppo di un protocollo di editing genetico per modulare il contenuto di asparagina libera nei semi di grano duro. Questo lavoro è stato svolto in collaborazione con il gruppo di ricerca del Prof. Nigel G. Halford presso Rothamsted Research, (Harpenden, Hertfordshire, UK). Durante un periodo trascorso presso Rothamsted Research, sono stati progettati tre RNA guida (gRNA) per l'editing basato su CRISPR/Cas9 con lo scopo di bersagliare il primo esone del gene *TdASN2*, un gene chiave nella sintesi dell'asparagina e che si esprime nelle ultime fasi dello sviluppo del seme. Dopo l'assemblaggio dei vettori, è stata applicata la tecnologia del *particle bombardment* per veicolare il DNA plasmidico all'interno di embrioni immaturi di frumento duro. Sono stati bombardati più di 700 embrioni immaturi e 10 piante sono state rigenerate, due delle quali sono risultate trasformate. L'attività è tuttora in corso e i risultati finora ottenuti hanno evidenziato la

necessità di ottimizzare i mezzi di selezione per la rigenerazione e la selezione dei trasformanti.

Chapter 1. General Introduction

1.1 Acrylamide

Acrylamide (C_3H_5NO) is a white, odourless, water-soluble crystalline organic compound that is widely used in its polymeric form, polyacrylamide.

Some examples of daily uses of polyacrylamide are related to its use in processes like papermaking, gas and oil industries etc. One of the primary applications of polyacrylamide is due to its ability to flocculate solid compounds in a liquid state and is applied in water and sewage treatment. Polyacrylamide is widely used in biochemistry, genetics, molecular biology and biotechnology for gel electrophoresis, a technique that allows separation of a wide range of biological molecules, including RNA (Petrov et al., 2013). Polyacrylamide may contain monomeric acrylamide as impurity. Acrylamide is considered a serious health hazard with acute toxicity and for this reason a potential water pollutant.

Acrylamide is known to be toxic to the nervous and reproductive systems, and suspected to be carcinogenic (Friedman et al., 2003; CONTAM Panel, 2015). Hence, it has been classed as a Group 2A carcinogen (probable human carcinogen) by the International Agency for Research on Cancer (IARC, 1994), the specialised cancer agency of the World Health Organization (WHO). WHO sets as acceptable the limit of $0.5\text{ }\mu\text{g}$ acrylamide per litre of water, or parts per billion ($\mu\text{g/kg}$) (WHO GUIDELINES FOR DRINKING-WATER QUALITY 4th edition, 2017).

Acrylamide can be inhaled, rapidly absorbed through the skin or, when ingested, absorbed from the gastro-intestinal tract, and subsequently spread to all tissues in the body. Once absorbed, it is metabolised to glycidamide ($C_3H_5NO_2$), an organic molecule which contains reactive electrophilic epoxide functional groups. Glycidamide forms adducts with DNA both *in vitro* and *in vivo* in human and animal tissues (CONTAM Panel, 2015) and gives a positive response in the Ames/Salmonella mutagenicity assay, which indicates glycidamide as possible DNA mutagen agent (Bergmark et al., 1991).

Glycidamide can be then considered the responsible for part (if not all) the acrylamide genotoxicity effect.

Reactions of acrylamide/glycidamide with proteins is a common marker for acrylamide exposure. A protein that commonly reacts with acrylamide/glycidamide is haemoglobin in the blood, where acrylamide forms the adduct N-(2-carbamoyl) valine (CEV) by reaction with valine residues located at the N-termini of globin chains.

Major information regarding acrylamide and its interaction with human body comes from smoker/non-smokers studies, since it is well known that acrylamide is a component of cigarette smoke. Another important source of information comes from studies conducted by Bergmark et al. (1993), which showed higher level of these adducts in the body of factory workers manufacturing acrylamide than in controls (Bergmark et al., 1993).

Moreover, Bergmark and colleagues (1997) noted high levels of CEV adducts in laboratory workers with PAGE gels compared to controls. Surprisingly, they observed in control samples levels of CEV adducts higher than expected. The authors hypothesised that other sources of acrylamide can contribute to CEV adducts in blood (Bergmark et al., 1997).

Later, Tareke et al. (2000) detected CEV adducts in laboratory rats fed with fried animal feeds and hypothesised that frying process could be a generator of acrylamide (Tareke et al., 2000).

The hypothesis was that foods contributed to the introduction of acrylamide into the organism of non-smoking workers, a hypothesis confirmed a couple of years later when the same research group reported the presence of acrylamide in various foods (Tareke et al., 2002).

As no acrylamide was detected in raw or boiled foods, acrylamide was classified as a food processing contaminant (Curtis et al., 2014). Food processing contaminants are generated in foods during industrial processes or cooking. In this context, acrylamide represents one of the food safety issues which has catalysed the greatest efforts in recent years (Friedman et al. 2008; EFSA Scientific Report, 2017; Rifai et al., 2020; Maan et al., 2022).

1.2 Acrylamide in foods

1.2.1 Acrylamide formation

Acrylamide formation in foods is due to Maillard reaction cascade which involve free (soluble and non-protein) asparagine ($C_4H_8N_2O_3$) and reducing sugars (Mottram et al., 2002; Stadler et al., 2002). In 1912 these cascade reactions were described for the first time by a French chemist, Louis Camille Maillard (Maillard 1912), from whom the reaction takes its name, but only in 1953 the currently considered scheme-reaction was proposed (Hodge 1953).

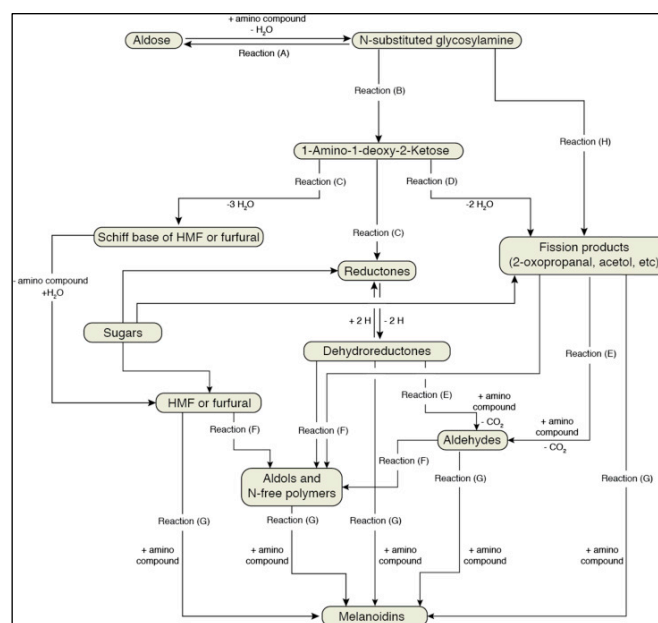


Figure 1.1 Maillard reaction cascade scheme; from Handbook of Food Chemistry (Martins et al., 2000).

Maillard reaction is a complex sequence of non-enzymatic reactions starting from reducing sugars, such as glucose, fructose and maltose, and free amino acids, such as asparagine, which occurs under high temperature processes ($T > 120^\circ C$) and low-moisture conditions. These conditions are typical of some cooking techniques, such as frying, roasting, baking and toasting, which are daily used to prepare food both at home and in commercial activities such as restaurants.

Hodge (Hodge 1953) schematically described the Maillard reaction cascade in three phases:

- the first phase involves amino compounds and reducing sugars, and terminates with the formation of Amadori or Heyns compounds;
- the second phase includes the formation of a series of intermediate compounds such as Strecker compounds;
- the third phase includes different pathways that lead to the condensation of intermediates compounds to form melanoidins, which confer colour and aroma to the final products.

Considering the Maillard reaction cascade, the key steps leading to the formation of acrylamide start from the generation of a Schiff base through the reactions between the amino group of free asparagine and the carbonyl group of the reducing sugar. The Schiff base undergoes a rearrangement of the double bonds, mediated by cyclisation and acid-catalysed rearrangement, which leads to the formation of an Amadori or Heyns compound, depending on whether the sugar is an aldose or a ketose, respectively.

This generates various products, from sugar dehydration and fragmentation, which participate in further reactions, such as a Strecker degradation.

Acrylamide forms from Strecker-type degradation of asparagine (Zyzak et al., 2003).

Decarboxylated Schiff base is then decomposed by removal of a carbonyl group, through a production of an intermediate (3-aminopropionamide) which is converted in acrylamide by the elimination of ammonia group (Granvogl et al., 2006; Granvogl et al., 2007; Hedegaard et al., 2008).

From this it can easily be understood that free asparagine and reducing sugars are both responsible for acrylamide-forming potential in foodstuffs.

In cereal grain-based foods, free asparagine concentration is the rate-limiting factor for acrylamide formation (Muttucumaru et al., 2006; Granvogl et al., 2007; Curtis et al., 2009; Curtis et al., 2010; Postles et al., 2016; Curtis et al., 2016). In wheat, for example, Halford et al. (2007) demonstrated a correlation

between free asparagine concentration in flour and acrylamide generated after heating samples reporting an R^2 values of 0.998.

1.2.2 Acrylamide levels in foods

Soon after the first detection of acrylamide in foodstuffs (Tareke et al., 2002), the European Commission's Joint Research Centre's Institute for Reference Materials and Measurements started monitoring the levels of this compound in foods.

Since 2007 monitoring of food acrylamide started being performed under Commission Recommendation 2007/331/EC which was then followed by Recommendation 2010/307/EU. Member States collect data on acrylamide levels in foods and provide them to EFSA (European Food Safety Authority), which analyses data and publishes annual reports (EFSA's 11th Scientific Colloquium, 2008; EFSA Scientific Report, 2009; EFSA Scientific Report, 2010; EFSA Scientific Report, 2011, EFSA Scientific Report, 2012) with the aim to inform the European Commission.

In 2015 the EFSA Scientific Panel on Contaminants in the Food Chain published a scientific opinion on acrylamide in food (CONTAM Panel, 2015), which includes data provided by six food associations.

From these reports emerged that highest acrylamide concentration was detected in chicory-based coffee substitutes, with an average concentration of 2942 $\mu\text{g}/\text{kg}$. In analysing acrylamide concentration in foods, EFSA scientific panel also considered the frequencies of assumption of foods and evaluated the risk related to daily acrylamide intake. As an example, acrylamide concentration in dry coffee was approximately 500 $\mu\text{g}/\text{kg}$, about 6 time less in comparison with chicory-based coffee, but the more frequent assumption of the former compared to the latter makes dry coffee riskier than chicory-based coffee. Hence, vegetable crisps and coffee substitutes were not identified as the biggest contributors to dietary acrylamide intake. In contrast, bread is considered the second major contributor to dietary acrylamide intake, despite

being relatively low in acrylamide, because it is regularly consumed and often toasted, thus increasing acrylamide level from tens to several hundred $\mu\text{g}/\text{kg}$. Mean values around hundreds of $\mu\text{g}/\text{kg}$ of acrylamide were measured for potato crisps, chips (fries), biscuits, breakfast cereals and crispbreads (Fig 1.2).

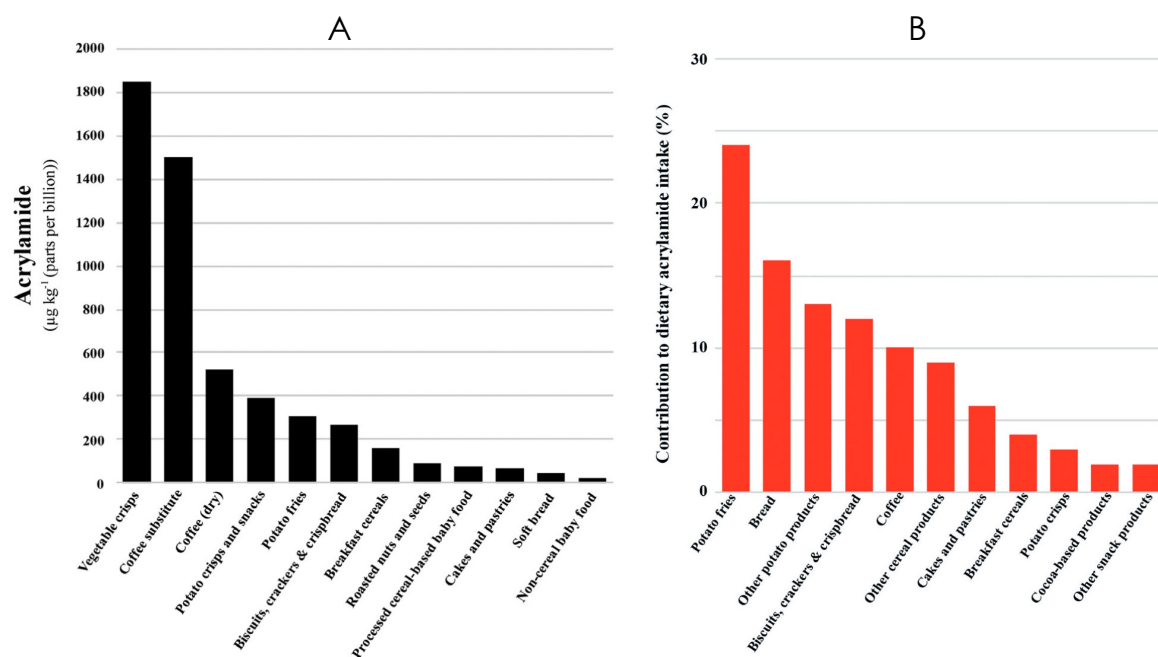


Figure 1.2: Acrylamide levels in different food groups (Panel A). Percentage contribution of different foods groups to daily dietary intake in Europe (Panel B). Figure Taken from Raffan and Halford (2019).

1.3 Factors affecting free asparagine accumulation in wheat

Free amino acids generally account for about 5% or less of the total nitrogen content of wheat grain, with free asparagine accounting for 10% or less of the total (CONTAM Panel, 2015). Consequently, this amino acid was of little scientific interest until the demonstration that it is a precursor of acrylamide during cooking. In recent years, many studies have compared the level of free asparagine in the seeds of different wheat genotypes to identify the factors influencing this trait.

In wheat grain, a high variability in free asparagine accumulation among wheat genotypes was demonstrated (Taeymans et al., 2004; Claus et al., 2006; Corol et al., 2006; Navrotskyi et al., 2018; Curtis et al., 2018b). Free asparagine concentration in the grain is affected by several factors. It is well demonstrated

that this trait is highly influenced by environmental factors, such as abiotic and biotic stresses (Lea et al., 2007), and by agronomic practices, including sulphur or nitrogen availability (Claus et al., 2006; Muttucumaru et al., 2006; Lea et al., 2007; Granvogl et al., 2007; Curtis et al., 2009, 2014, 2018b; Martinek et al., 2009; Raffan et al., 2020; Wilson et al., 2020), and poor disease control (Martinek et al., 2009; Curtis et al., 2016). In addition, genetic factors may influence this trait, since the accumulation of free asparagine in wheat seed is a specific trait showing good heritability (Hedegaard et al., 2008; Muttucumaru et al., 2006).

1.3.1 Role of environmental factors and crop management practices in the modulation of asparagine levels in wheat

Environmental factors can directly influence asparagine metabolism and the nutritional status of wheat plants has a high impact on free asparagine levels in the grain (Muttucumaru et al., 2006; Granvogl et al., 2008). Therefore, appropriate field management strategies could be used to influence asparagine metabolism, and subsequently free asparagine accumulation.

In 1980 Winkler and Schön (Winkler and Schön, 1980) first reported the impact of nitrogen fertilization on amino acid composition demonstrating that high levels of nitrogen supply resulted in higher levels of free amino acids, including asparagine. They found increasing free asparagine levels with increasing grain nitrogen concentration in barley.

It is known that nitrogen is transported into the seed and exceeding nitrogen, not required for the synthesis of structural and metabolic proteins, is utilised to provide a store for germination and seedling growth.

According to the study, late nitrogen fertilization treatments may have led to an increased of both crude protein and free asparagine, which is an important transported and stored form of nitrogen.

In contrast, it has been demonstrated that severe sulphur deficiency is linked to an increase in free asparagine (Shewry et al., 1983, Claus et al., 2006; Martinek et al., 2009;). Storage proteins can be classified into two main groups according to their relative contents of the sulphur-containing amino acids, cysteine and methionine. Under sulphur-deficient growing conditions, the

plant cannot produce proteins rich in sulphur amino acids and the balance is shifted to the fraction of proteins that contain a small amount of cysteine and methionine or none at all. This leads to an extra amount of not incorporated amino acid, such as asparagine.

Total concentration of amino acids was found to be higher than in controls, while concentration of free asparagine was up to 30 times higher in conditions of severe sulphur deficiency compared to control conditions (Muttucumaru et al., 2006).

The evidence reported by Muttucumaru and collaborators in 2006 (Muttucumaru et al., 2006) have been confirmed by subsequent work which highlighted the relationship between sulphur deprivation and the increase in free asparagine in wheat grains (Granvogl et al., 2007; Curtis et al., 2009; Curtis et al., 2014a; Curtis et al., 2018b).

As a result of these studies, European commissions with Regulation (EU) 2017/2158 informed farmers to 'maintain balanced sulphur levels in the soil and to ensure correct nitrogen application' (Commission Regulation, 2017).

Biotic stress has also been shown to increase asparagine accumulation in many plant species (Lea et al., 2007). Curtis and collaborators found that serious infections lead to increased concentration of free amino acids, including free asparagine (Curtis et al, 2016). In the same study, variability in free asparagine increase in response to pathogen attack among wheat varieties was reported, the increase ranging from 17% to 135%. Other studies strengthened the link between pathogen attack and free asparagine levels (Wath et al., 2015; Curtis et al., 2016; Navrotskyi et al., 2018). Relatively little is known about the link between free asparagine concentration and pathogen infection. Some authors hypothesized that free asparagine may be involved in a signaling pathway in response to pathogen infections.

One candidate signalling factor of this possible pathway is represented by the general control non-derepressible-2 (GCN2)-type protein kinase, (Hey et al., 2010) which has been shown to affect asparagine synthetase gene expression in wheat seedlings (Byrne et al., 2012). Yeast GCN2 has also been shown to

interact with a yellow rust R-protein (Yr10), whereas barley HvGCN2 gene expression increases in the first 12 h after inoculation with the pathogen (Yildirim-Ersoy et al., 2011).

A second candidate is another protein kinase, sucrose nonfermenting-1-related protein kinase-1 (SnRK1). SnRK1 coordinates nutrient availability and stress and hormonal signalling, maintains cellular energy balance (Hey et al., 2010; Baena-González et al., 2007) and is directly implicated in the control of asparagine synthetase gene expression in *Arabidopsis thaliana* (Baena-González et al., 2007). Recently, it has been shown to interact with the protein encoded by *TaFROG*, a gene induced in wheat by *Fusarium graminearum* infection, (Perochon et al., 2015) and to become activated in wheat in response to *Septoria tritici* infection (Yang et al., 2013).

Therefore, biotic stress mitigation is an important strategy in view of limiting free asparagine accumulation in wheat grain. For this reason, practices for the management of fungal diseases were included within the European Commission Regulation (EU) 2017/2158.

1.3.2 Influence of genetic factors on free asparagine accumulation in wheat

In recent years, the sequencing of multiple wheat genomes (Walkowiak et al., 2020) and the development of numerous marker technologies (Wilkinson et al., 2020) have provided new tools in the field of genetic research.

Wheat breeding and research has been greatly facilitated in recent years by the sequencing of wheat genomes (Walkowiak et al., 2020). Both bread (cv. Chinese Spring; International wheat genome sequencing consortium, 2018) and durum (cv. Svevo; Maccaferri et al., 2019) wheat genome sequences have been reported. These valuable genetic resources have resulted in an ever-increasing development of marker technologies (Wilkinson et al., 2020) and have allowed researchers to take a step forward in understanding the influence of genetic factors controlling specific traits, including free asparagine accumulation in wheat grains.

A first work related to the genetics of free asparagine accumulation was reported by Emebiri and collaborators (2014), who performed an analysis on 92 bread wheat genotypes and identified a region on chromosome 5A associated with free asparagine content. They also found differences among varieties in free asparagine concentration, varying approximately 3-fold between the low and high free asparagine accumulator genotypes (Emebiri et al., 2014).

Some years later, a genome wide association study (GWAS) was performed by Rapp and collaborators (2018), who found QTLs associated to free asparagine accumulation and estimated a heritability about 0.65 for this trait.

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Chapter 2. Aim of the thesis

The general aim of this PhD thesis was to study the natural variation of free asparagine levels in durum (*Triticum turgidum* L. ssp. *durum* (Desf.) and bread (*Triticum aestivum* L.) wheat grains, and to identify candidate genes involved in the control of free asparagine accumulation in wheat seeds.

Three research tasks related to these goals were carried out.

The first task aimed to explore genetic diversity related to free asparagine content in durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) and to identify candidate genes putatively associated to free asparagine accumulation in seeds.

To this purpose, 201 durum wheat genotypes, which originate from 33 different countries, were selected from an international collection (the Global Durum Panel) which consists of a wide representation of *Triticum turgidum* ssp. *durum*. To increase genetic variability, a large number of durum wheat landraces were included in the study. The 201 genotypes were sown in experimental fields for three years and free asparagine level was measured in wholemeal flour obtained from harvested grains. With these phenotypic data, a multi-locus genome-wide association study (m-GWAS) was applied to identify chromosomal regions and candidate genes involved in free asparagine accumulation in wheat seeds.

Analysis and results related to this task are reported in Chapter 3 of this thesis and are part of a manuscript in preparation, which will be submitted to an international peer-review journal.

The second task aimed to characterise elite bread wheat (*Triticum aestivum* L.) varieties, relevant for Italian market, to identify genotypes showing stable accumulation of free asparagine across growing year/location and contrasting for high/low free asparagine accumulation.

The study included 54 bread wheat varieties from the Italian “National Network of Common Wheat Variety Comparison” (In Italian: “Rete Nazionale di Confronto Varietale del Frumento Tenero”) coordinated by Council for Agricultural Research and Economics (CREA). Seeds from six field trials in three Italian locations over two years were collected and wholemeal flours were analysed for free asparagine concentration. Four genotypes were selected for their contrasting levels of free asparagine accumulation across years and cultivation environments. The acrylamide-forming potential of these selected genotypes was evaluated. In particular, the selected genotypes were analysed for technological properties and used to prepare wood-baked pizza in which acrylamide generated was quantified.

The data are presented in two scientific articles, the first published in 2023 in the international journal *Plants* (Chapter 4 of the PhD thesis), while the second was submitted to the international journal *Journal of the Science of Food and Agriculture* (Chapter 5 of the PhD thesis).

The third task had the goal to set up a gene editing protocol in durum wheat CRISPR/Cas9 technology.

This activity was developed within the framework of a collaboration with the research group of Prof Nigel G. Halford at Rothamsted Research in Harpenden (Hertfordshire, UK), who is a recognised expert in the study of free asparagine content related to acrylamide forming potential in wheat.

In the frame of this activity, I spent five months in the laboratory of Prof. Halford to set up a transformation and gene editing protocol in durum wheat, cv. Svevo. In particular, the CRISPR/Cas9 experiment was designed to target the first exon of *TdASN2* gene, a key gene in asparagine synthesis which is expressed at late stages of seed development.

The activity is still ongoing, and the results are reported in Chapter 6 of this thesis.

Chapter 3. Genome Wide Association Study of Free Asparagine Content in Durum Wheat (*Triticum turgidum* L. ssp. *Durum* Desf.) Grain

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The results presented in this chapter have been included in the article:

Tafuri A., Pirona R., Fricano A., Mazzucotelli E., Ravaglia S., Giordano M., Zuccaro M., Cagliani L. R., Consonni R., Ceriotti A., Baldoni E., 2023. Genome Wide Association Study of Free Asparagine Content in Durum Wheat (*Triticum turgidum* L. ssp. *Durum* Desf.) Grain. (In preparation).

Abstract

Acrylamide, a molecule which is known to be neurotoxic and suspected to be carcinogenic, has been detected in many foods after high temperature processing. In wheat derivatives, free asparagine content has been identified as a key factor in acrylamide formation. Therefore, the control of free asparagine levels in wheat seeds is of considerable interest in contemporary crop and food sciences. Our study aims to explore the natural variation in free asparagine levels in durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.)) grains to identify candidate genes controlling this trait. To this purpose, 201 durum wheat genotypes (including 193 landraces) were selected from the international Global Durum Panel (GDP), a collection which has been previously genotyped using Illumina iSelect 90K SNP array technology. Population structure analysis indicated that the selected panel maintained a high level of genetic variability, comparable to that observed for the original GDP population. Clustering analysis identified four groups, matching the geographic region of origin. Linkage disequilibrium (LD) analysis showed that in our collection LD extends till 4.4 Mbp. The 201 genotypes were sown in an experimental field located in Fiorenzuola d'Arda (Italy) for three years. The field experiments were established in a randomized complete block design with five check cultivars, each replicated seven times. Wholemeal flour was obtained from harvested grains and free asparagine content was measured using an enzymatic method. The analysis highlighted a good variability for free asparagine levels in our subcollection. An analysis of 100 selected samples through High Resolution Proton Nuclear Magnetic Resonance (¹H-NMR) spectroscopy showed a good correlation between the values obtained with the two methods. A multi-locus genome-wide association study was performed to identify genomic regions associated with free asparagine content. Six Single Nucleotide Polymorphisms (SNPs) on chromosomes 6A, 7A, 2B, 4B, 7B associated with grain free asparagine content were identified. A total of 396 genes are located in LD blocks and will be considered for future studies.

3.1 Introduction

Chemical contaminants are substances that are unintentionally present in foods. These compounds may be present in foods as a result of different stages of their production and the most relevant include natural toxin (alkaloids, mycotoxins etc.), environmental contaminants (dioxins, persistent chlorinated pesticides, cadmium etc.) and process contaminants which are generated in foods during industrial processes or cooking. In the category of process contaminants, acrylamide (C_3H_5NO) represents one of the food safety issues which has catalysed the greatest efforts in recent years. Acrylamide is a white, odourless, water-soluble molecule and is rapidly absorbed through the skin and from the gastro-intestinal tract. Once metabolised, it produces glycidamide ($C_3H_5NO_2$) which forms adducts with DNA both in vitro and in vivo in human and animal tissues (CONTAM 2015). This behaviour implicated its classification in group 2a as a probable carcinogenic to humans by the International Agency for Research on Cancer (IARC; IARC 1994). Acrylamide is generated during the technological process of baking, frying, grilling and in general in those processes at temperature higher than $120^{\circ}C$ and in presence of low moisture. The reactions leading acrylamide formation are part of Maillard cascade reactions and involve free (soluble, non-protein) asparagine ($C_4H_8N_2O_3$) which reacts with reducing sugars to form acrylamide (Mottram 2002, Stadler 2002). In cereal grain-based foods, free asparagine concentration is the rate-limiting factor for acrylamide formation (see Raffan 2019 for review). Surdyk and collaborators (2004) set an experiment to investigate internal and external factors affecting acrylamide formation in yeast-leavened bread. Bread wheat cultivars low in free asparagine and reducing sugars were selected and flours were used to prepare bread. The researchers added increasing equimolar levels of these compounds to the dough and compared the acrylamide levels after baking at $270^{\circ}C$ for 15 or 20 minutes with control bread without added asparagine and sugar. They found that the addition of asparagine markedly increased the acrylamide content of bread, while added fructose did not affect its content. They also

investigated the role of temperature and baking time in a separate experiment, reporting that baking temperatures above 200 °C and baking duration increase the acrylamide content in bread.

Consistently, Halford et al. (2007) demonstrated a correlation between free asparagine concentration in wheat flour and acrylamide formation after heating, reporting an R^2 values of 0.998. As asparagine is the rate-limiting factor in the reaction that generates acrylamide, another aspect that is under study is related to factors that regulate its amount in wheat seeds.

It was demonstrated that high levels of nitrogen fertilization induce higher free asparagine in the grain (Winkler and Schön, 1980) resulting in higher levels of acrylamide in food products.

Another research group demonstrated an opposite regulation in response to sulphur supply, reporting an increase in free asparagine accumulation under sulphur starvation (Shewry et al., 1983). Further studies confirmed that both nitrogen and sulphur fertilisation practices play an important role in determining free asparagine levels (Raffan et al., 2020; Wilson et al., 2020; Curtis et al., 2018; Curtis et al., 2014; Curtis et al., 2009; Martinek et al., 2009; Granvogl et al., 2007). These findings, as well as the implication on human health of the presence of high asparagine levels in wheat flours, induced the European commissions to inform farmers to 'maintain balanced sulphur levels in the soil and to ensure correct nitrogen application' through the Regulation (EU) 2017/2158 (European commission, 2017).

More recently, Curtis and collaborators (2018) identified eight bread wheat varieties that accumulated consistently low free asparagine concentration in the grain, regardless of crop management, including sulphur deprivation, and growing year.

In addition, biotic stresses have been shown to play an important role in free asparagine accumulation in wheat grain (Lea et al., 2007). Martinek and collaborators (2009) reported that an efficient fungicide treatment may decrease asparagine concentration (Martinek et al., 2009). This evidence was supported by another research group, that demonstrated a correlation

between the onset of serious diseases and an increase in asparagine concentration and reported a different response to pathogen attack among wheat varieties (Curtis et al., 2016).

These results highlight a varietal difference in free asparagine accumulation and point out an important role of the genetics in controlling this trait.

Wheat breeding and research has been greatly facilitated in recent years by the sequencing of the genomes of both bread (cv. Chinese Spring; International wheat genome sequencing consortium, 2018; Walkowiak et al., 2020) and durum (cv. Svevo; Maccaferri et al 2019) wheat.

Genomes sequencing has given rise to an ever-increasing development of marker technologies, thus helping researchers to better understand the genetic bases of complex traits, including free asparagine accumulation in wheat grains (Wilkinson et al., 2020).

The first relevant genome wide study related to the genetic control of free asparagine accumulation in wheat was reported by Emebiri and collaborators (2014). The authors performed a genetic variation analysis in 92 wheat genotypes and identified a region in chromosome 5A associated with free asparagine content. They also found some differences among genotypes in free asparagine concentration, varying approximately 3-fold between the lowest and highest free asparagine accumulator genotypes (Emebiri et al., 2014).

Some years later, a genome wide association study (GWAS) was performed by Rapp and collaborators (Rapp et al., 2018), who reported interesting QTLs associated with free asparagine accumulation with high heritability.

In the last years, different technological methods were proposed to reduce acrylamide-forming potential, as reported into the 'Acrylamide Toolbox' by Food Drink Europe (2019). The substitution of NH_4HCO_3 with alternative raising agents (sodium bicarbonate and acidulants, sodium bicarbonate and disodium diphosphates with organic acid) was demonstrated to be an effective way to lower acrylamide formation in bakery products. Unfortunately, changes in flavour, colour and texture of several products have been reported

when these substitutions of raising agents were applied (Food Drink Europe, 2019). Another proposed solution is the use of asparaginase (enzyme which depletes asparagine) in dough to decrease asparagine levels before baking. Unfortunately, despite some of these have been successfully applied, they are not applicable to all foods, since this method is very expensive and can affect product quality. The food industry would therefore benefit from the availability of cereal grains with lower contents of free asparagine and consequently with reduced acrylamide-forming potential. To pursue this goal, the analysis of the genetic bases underlying free asparagine accumulation in wheat grain could provide fundamental knowledge and tools for breeding programs to develop new varieties with reduced free asparagine content without losing in food quality. Particularly, the use of multi-locus models to perform a GWAS, named mGWAS, is a new tool to dissect complex trait such as free amino acid accumulation (Peng 2018).

The aim of this study was to explore the genetic variability of free asparagine accumulation in durum wheat (*Triticum turgidum* L. ssp. *Durum* (Desf.)) to identify molecular markers statistically associated to this trait. To pursue this objective, two hundred durum wheat genotypes, originating from 33 different countries, were selected from an international collection, and sown in experimental fields for three years. Free asparagine in wholemeal flour obtained from harvested grains was measured and GWAS was performed to identify markers associated to free asparagine content.

3.2 Materials and Methods

3.2.1 Plant Material and field trials

Two-hundred-one genotypes originating from 33 worldwide countries (Table S3.1) were selected from The Global Durum Wheat Panel (GDP, Mazzucottelli et al., 2020) to assemble our study-population, thus consisting of a wide representation of *Triticum turgidum* ssp. *durum* with most landraces (180 landraces and 21 cultivars) to maximize genetic diversity.

All accessions were grown at Fiorenzuola d'Arda (Piacenza, Italy) at Council for Agricultural Research and Economics-Research Centre for Genomics and Bioinformatics (CREA-GB). Field trials were performed during the 2019–2020, 2020–2021, 2021–2022 cropping seasons (Table 3.1).

Table 3.1: Geographical coordinates, sowing and harvesting dates related to all agronomic seasons.

Year	Location	field coordinates	Sowing date	Harvesting date
2019/2020	Fiorenzuola d'Arda	44°55'39.1"N	12 November 2019	7 July 2020
		9°53'40.7"E		
2020/2021	Fiorenzuola d'Arda	44°55'39.1"N	30 October 2020	8 July 2021
		9°53'40.7"E		
2021/2022	Fiorenzuola d'Arda	44°55'39.1"N	24 November 2021	5 July 2021
		9°53'40.7"E		

Field trials were conducted following an augmented experimental design created using agricolae (R package version 1.4. 0, Mendiburu and Muhammad Yaseen, 2020) with five check cultivars (Meridiano, Kiperound, Kirmizi, Mindum, Iran1) that were each replicated seven times. Each plot contained 34 rows of 1 m each one spaced 70 cm apart, with 25 plants per row. Field trials were carried out following standard agronomic wheat management practice (Table S3.2). Fungicide and fertilizers were provided in-season, following optimal local management practices. Weather data was recorded for all years (Table S3.3) and soil composition analyses were performed before sowing (Table S3.4).

For each field trial, mature seeds were collected from the entire rows and stored for the analysis.

3.2.2 Chlorophyll measurements

Chlorophyll was measured with an optical chlorophyll meter (MC-100, Apogee Instruments, Logan, UT) which was calibrated for durum wheat species according to manufacturer instructions. The MC-100 calculates a ratio between transmission of radiation at 931 nm divided by transmission of

radiation at 653 nm, the result being called the Chlorophyll Content Index (CCI) which was automatically converted into μmol of chlorophyll per m^2 by the instrument's software.

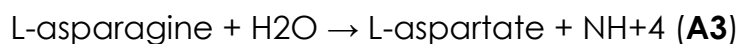
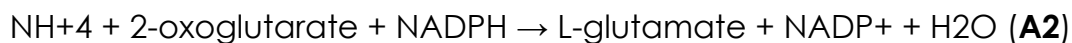
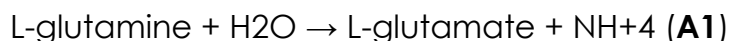
Individual measurements were made on five different plants per row for the selected 45 genotypes on 20 mm^2 of a circular surface with 5 mm diameter, following manufacturer instruction. Single measurements were performed for a total of 10 measurements for each flag leaf (5 on top surface and 5 on bottom surface of the leaf). Each measurement was taken in a specific part of the leaf, *i.e.*, from one third to one half of the distance along the leaf, from the stem insertion. The first measurement was taken about 10 days after heading and subsequent measurements at regular intervals every 2-3 days until complete senescence of the plants during the second and the third crop seasons.

3.2.3 Free asparagine quantification

Quantification of free asparagine levels in the whole-grain wheat samples was conducted using a spectrophotometric analysis after total deproteinization of samples, as described in Tafuri et al. (2023). Briefly, whole-grain samples were ground through a 0.5 mm grid diameter mesh using a laboratory mill (Cyclotec CT293, FOSS, Hilleroed, Denmark) to obtain a homogeneous powder. Deproteinization was performed on wholemeal flour (5 g) by addition of 15 mL of 1 M perchloric acid and shaken for 30 min at room temperature. The suspension was centrifuged at 5300 RCF for 3 min at 4°C to produce a clear extract. Clear supernatant was brought to pH 8 by adding 2 M KOH and adjusted to 50 mL in a graduated cylinder.

Then, 200 μL of the 50 mL-deproteinized sample were used for free asparagine quantification using the L-Asparagine/L-Glutamine/Ammonia Assay Kit (K-ASNAM, Megazyme, Wicklow, Ireland).

This kit foresees the use of three enzymatic reactions.



The absorbance was recorded using a Jasco V-630 spectrophotometer (JASCO, Japan), after each of the 3 reaction steps. The concentration of free asparagine in the sample was calculated as reported by the manufacturer and briefly reported below.

CALCULATION:

Determine the absorbance differences (A2-A3) for both blanks and samples. Subtract the absorbance difference of the blank from the absorbance difference of the corresponding sample, thereby obtaining the change in absorbance (ΔA) resulting from the analyte in question, as follows:

$$\Delta A_{\text{L-asparagine}} = (A2-A3)_{\text{sample}} - (A2-A3)_{\text{blank}}$$

The concentration of L-asparagine can be calculated as follows:

$$c(g/L) = \frac{V * MW * \Delta A}{\epsilon * d * v}$$

where:

V = final volume [mL]

MW = molecular weight of analyte [g/mol]

ϵ = extinction coefficient of NADPH at 340 nm = 6300 [l x mol⁻¹ x cm⁻¹]

d = light path [cm]

v = sample volume [mL]

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

$$c(g/100g) = \frac{c}{\text{weight sample}} * 100$$

These calculations can be simplified by using the Megazyme Mega-Calc™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

3.2.4 Procedure for computing the adjusted means of free asparagine content.

All field trials were analysed using an augmented experimental design and adjusted means of free asparagine were computed using a mixed linear model considering genotypes as fixed effects. To integrate results across field trials carried out in 2020, 2021 and 2022 growing seasons, five check cultivars (Meridiano, Kiperound, Kirmizi, Mindum, Iran1) were included. Mixed linear models for analysing field trial data were implemented in lme4 package (Bates et al. 2015) along with R 4.0.3 (Core R Team 2019) and used to compute the best linear unbiased estimates (BLUEs) of genotypic effects using the following model:

$$y_{ijk} = \mu + \text{Gen}_i + \text{Year}_j + \text{Year}_j(\text{Block}_k) + e_{ijk}$$

where y_{ijk} is the response variable, that is the raw measurement of free asparagine, μ is the general mean, Gen_i is the fixed effect of the i^{th} genotype, $\text{Year}_j(\text{Block}_k)$ is the effect of the k^{th} incomplete block within the j^{th} year. In this model, it is supposed that the residual terms e_{ijk} are normally distributed with mean 0 and variance equals to σ^2 , that is $e_{ijk} \sim \text{NIID}(0, \sigma^2)$.

3.2.5 ¹H-NMR profiling

NMR sample preparation was carried out according to the procedures described in Ward et al. (2003) and Baker et al. (2006). NMR extractions into 4:1 D₂O: MeOD containing 0.05% d₄-trimethylsilylpropionate (TSP) (10ul into 700ul of sample).

All NMR spectra were recorded on Bruker AVANCE NEO 600 spectrometer (Bruker Biospin, GmbH Rheinstetten, Karlsruhe, Germania), operating at 14.07 T, equipped with a 5 mm reverse Z gradient cryoprobe Prodigy, and thermostated autosampler, at 300 K.

¹H NMR spectra were acquired with a monodimensional NOESY sequence with solvent presaturation scheme with 256 scans over 64K point of data, and a spectral width of 7800 Hz. An opportune relaxation time was used to allow a complete relaxation of all nuclei.

A resolution enhancement function (LB=0.3 Hz) was applied before Fourier transformation. All spectra were phased, baseline corrected and aligned with respect to TSP at 0.0 ppm (TOPSPIN software version 4.1.2, Bruker Biospin, GmbH Rheinstetten, Karlsruhe, Germania).

The quantification of free asparagine (β'CH at 2.95 ppm) in the extracts of all analysed samples, was carried out adopting the TSP signal as internal reference by using the following equation (Bharti & Roy, 2012):

$$\frac{M_x}{M_y} = \frac{I_x}{I_y} * \frac{N_y}{N_x}$$

where M, I and N indicate molarity, signal integral value, number of protons generating the selected signal for integration, of asparagine (X) and the reference standard used (Y=TSP).

3.2.6 Population STRUCTURE

Single nucleotide polymorphism (SNPs) data were downloaded from the GrainGenes database (<https://wheat.pw.usda.gov/GG3/>). Briefly, they were produced using the Illumina iSelect 90K SNP array technology (Wang et al.,

2014) and described by Mazzucotelli et al. (2020). The SNP data were filtered to remove markers with allele frequencies lower than 5%.

To establish the number of groups in our 201 samples population, a discriminant analysis of principal components (DAPC) was performed using the 'ade4' package 1.4-1 (Jombart et al., 2010) in RStudio 4.2.2 (R Development Core Team, 2022). The decreasing rate of the Bayesian information criterion (BIC) was visually inspected (Figure 3.7), then the number of clusters was selected taking the value of k above which BIC values decreased.

After cluster number selection, an analysis of admixture by kinship Bayesian clustering technique was used to classify the group of genetically similar population via statistical software STRUCTURE v.2.3 (Pritchard et al., 2000). Burn-in iterations of 10,000 cycles, followed by a simulation run of 100,000 cycles, the admixture model selection, and 4 as k value based on DAPC results were used with 11 independent runs.

3.2.7 LD Decay

Linkage disequilibrium (LD) was calculated using TASSEL5 and measured using r^2 . SNP dataset has been used to calculate pairwise marker correlation (r^2) for each chromosome, considering SNPs with an MAF > 0.05, using TASSEL5 (Bradbury et al., 2007).

LD decay curve was fitted using non-linear model (in Rexroad and Vallejo (2009)). To estimate the local LD value along chromosomes, each marker LD was calculated using the mean r^2 with the 50 nearest markers, and then smoothed as one value using the step-sliding window.

The LD decay was determined by plotting the r^2 values against the genetic distance of loci pairs (Mb) for each chromosome and a trend line describing the LD decay was calculated by locally weighted polynomial regression (LOESS) in R. The intersection point between the LD curve and the r^2 threshold determined the LD decay.

3.2.8 Phylogenetic tree

Population phylogenetic tree was estimated through Neighbor-Joining clustering based on Nei's genetic distances of SNPs data, using the poppr v. 2.9.4 and adegenet packages of R (Jombart, 2008; Kamvar et al., 2014; R Core Team, 2022) with 1000 bootstrap replications. The phylogenetic tree was visualized through ggtree package of R (Wang et al., 2020).

3.2.9 Association Analyses

Free asparagine levels were studied with Genome Association and Prediction Integrated Tool (GAPIT, Liptka et al., 2012, Tang et al., 2016), TASSEL5 (Bradbury et al., 2007) and mrMLM (Zhang et al., 2020) R package. This package includes six mGWAS models: mrMLM (multi-locus random-SNP-effect MLM, Wang et al., 2016), FASTmrMLM (Tamba et al., 2017), FASTmrEMMA (fast multi-locus random-SNP-effect efficient mixed model analysis, Wen et al., 2017) ISISEMBLASSO (iterative modified-sure independence screening expectation-maximization-Bayesian least absolute shrinkage and selection operator, Tamba et al., 2017), pLARmEB (polygenic-background-control-based least angle regression plus empirical Bayes, Zhang et al., 2017) pKWmEB (integration of Kruskal-Wallis test with empirical Bayes, Ren et al., 2018).

The threshold level for significant marker-trait associations was $-\log_{10}p = 3$ or above for GAPIT and TASSEL5.

A critical threshold for significantly associated SNPs was set at $LOD > 3.0$ for the six multi-locus models. Statistically significant SNPs detected in at least in two different algorithms were considered Quantitative Trait Nucleotides (QTNs) in our analyses and further analysed to seek putative candidate genes. According to LD block previously calculated a list of candidate genes was compiled using Ensembl Biomart (release 56 - Feb 2023) and include those genes located in LD blocks.

3.3 Results and discussion

3.3.1 Free asparagine quantification

Field trials of 201 genotypes of *Triticum turgidum* L. ssp. *durum* (Desf.) originated from 33 countries were performed for three years in Fiorenzuola d'Arda (PC, Italy). For each field trial, mature seeds were collected from the entire rows and wholemeal flour was obtained from harvested grains. Free asparagine quantification was carried out using an enzymatic assay on wholemeal flour samples according to Lecart et al. (2006) and Tafuri et al. (2023) and results are reported in Table S3.5. The free asparagine content in the considered samples ranged from 1.66 to 2.52 mmol/kg dry matter (d.m.) for 2019/2020, from 1.00 to 1.65 mmol/kg d.m. for 2020/2021 and from 0.89 to 1.61 mmol/kg d.m. for 2021/2022. Figure 3.1 shows the free asparagine measurements across the three years of field trials.

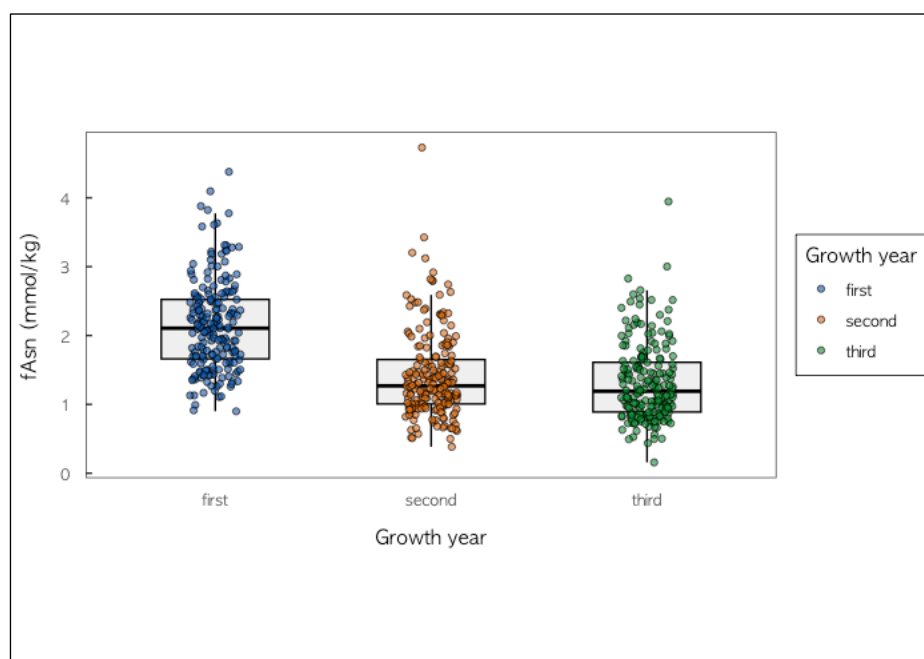


Figure 3.1: Box plots of free asparagine measurements from the three years field trials.

The median values of free asparagine level in the 201 genotypes were 2.11, 1.27 and 1.19 mmol/kg d.m. for the first, the second and the third year, respectively. An ANOVA test verified that the first year was significantly different from the other two years (Table S3.6). These data suggested that

growth conditions of the first year led to a higher accumulation of free asparagine in comparison with second and third-year field trials, where the ANOVA test resulted not significant (Table S3.6).

A total of 100 genotypes (50 genotypes showing the highest amounts and 50 genotypes showing the lowest amounts of free asparagine accumulated) from first year field trial were analysed by ^1H -NMR (Table S3.7) to measure free asparagine using a different assay and to collect data which will allow to obtain a more general view of amino acid metabolism in seeds. Pearson's correlation between values obtained by NMR and enzymatic assays was calculated in $R^2=0.9$ (Fig. 3.2). These results were in line with those obtained with the enzymatic method and thus confirmed the free asparagine levels measured of the 201 genotypes.

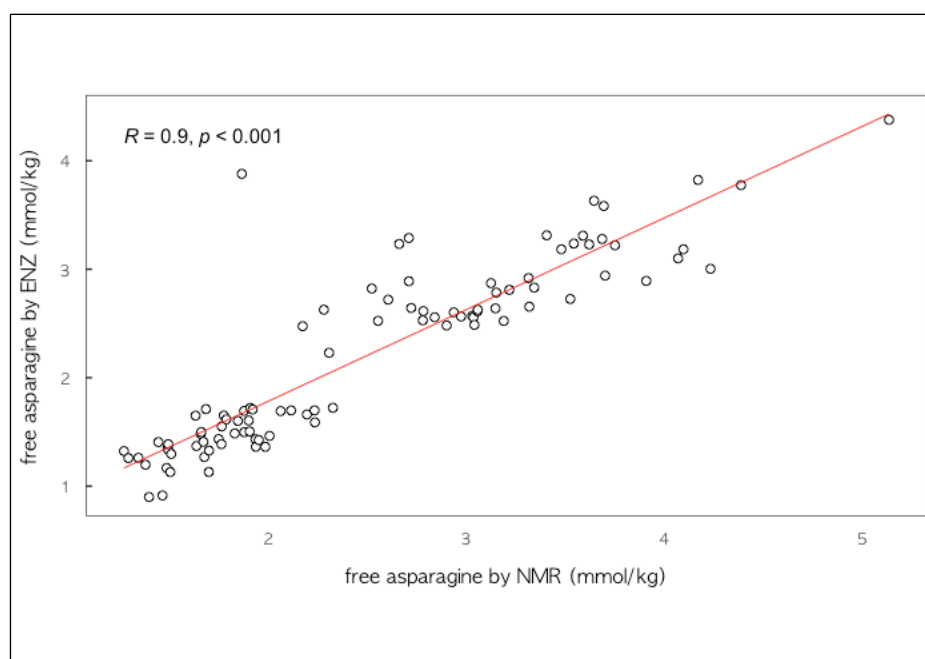


Figure 3.2: Scatter Plot showing Pearson's correlation between free asparagine measurements obtained via enzymatic method (Y axis) and NMR (X axis).

Crop management and other environmental factors, both alone and interacting with genetic factors (G X E), have a well-established impact on free asparagine accumulation (Ohm et al., 2017; Navrotsky et al., 2018; Raffan et al., 2019; Xie et al., 2021). It has been well demonstrated how differences in

growth location and/or growth year may influence free asparagine accumulation due to different environmental conditions, such as rainfall and soil water availability, or specific climatic factors (Muttucumaru et al., 2006; Granvogl et al., 2007; Corol et al., 2016; Curtis et al., 2018).

Many authors have shown that sulphur or nitrogen availability strongly affect free asparagine accumulation (Muttucumaru et al., 2006; Granvogl et al., 2007; Halford et al., 2007; Curtis et al., 2009; Martinek et al., 2009; Curtis et al., 2014; Raffan et al., 2020; Wilson et al., 2020). For this reason, we used the same standard protocol for nitrogen and sulphur input across years (Table S3.2) to minimise the influence of crop management. The observed higher values in free asparagine accumulation during the first year could be due to environmental factors. Corol et al., (2007) reported that the level of precipitations and temperatures during grain development (between heading and harvest) has the greatest effect on free asparagine concentration in mature grain.

Senescence time of the flag leaf and grain filling are two physiological processes that mostly affect the composition of wheat seeds (Uauy et al., 2006). To evaluate whether the observed differences in the amount of free asparagine correlate with seed size or senescence time of the flag leaf, we evaluated these parameters in relation to free asparagine levels. For senescence, we measured the chlorophyll content of the flag leaf of 45 selected genotypes during the grain filling stage (from heading to complete senescence of the plants) in the second and third year of field trials. Pairwise correlation coefficients were calculated using the Spearman's rank correlation method between the Day After Heading (DAH) when the chlorophyll content falls below 10% of the highest measured value and the measured free asparagine grain content (Figures 3.3 and 3.4).

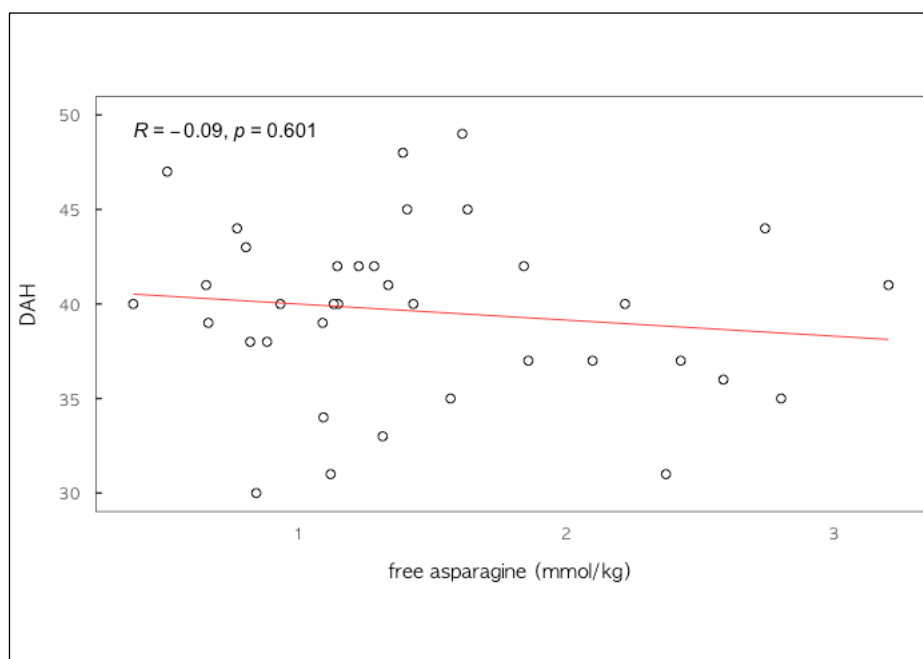


Figure 3.3: Scatter plot showing Spearman's correlation between DAH (Y axis) and free asparagine measurements (X axis). Data related to 2020/2021 agronomic year.

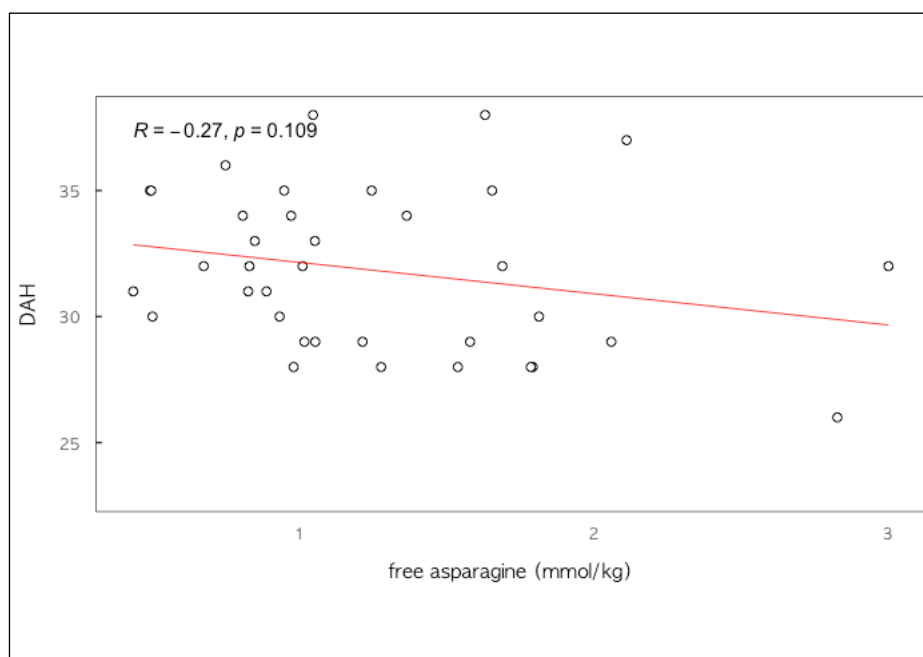


Figure 3.4: Scatter plot showing Spearman's correlation between DAH (Y axis) and free asparagine measurements (X axis). Data related to 2021/2022 agronomic year.

No correlation was found between the length of senescence period and the amount of free asparagine accumulated in the seeds. For grain filling, we measured the Thousand Kernel Weight (TKW) mean values of the three years of the 201 genotypes (Figure 3.5).

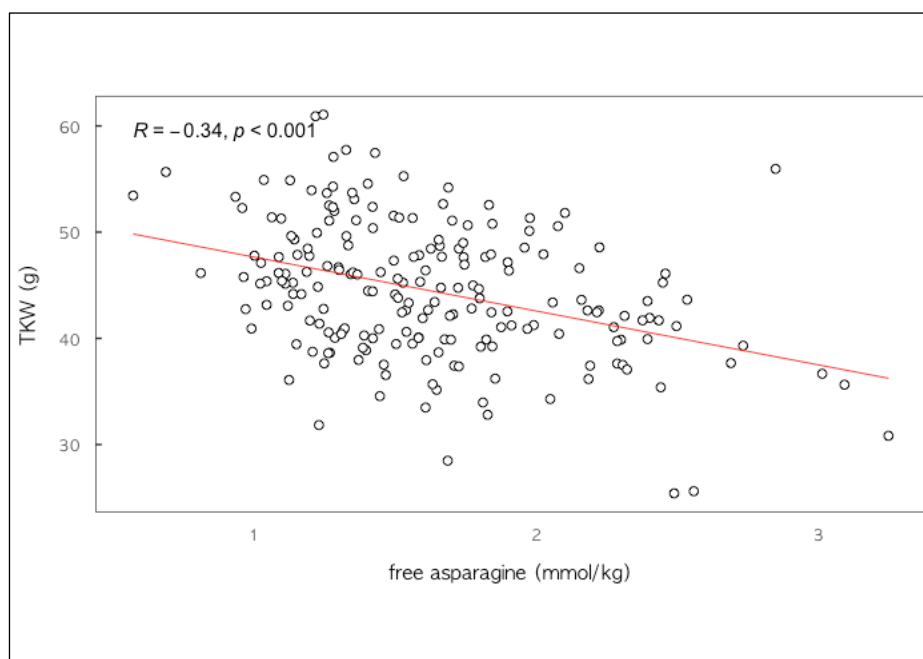


Figure 3.5: Scatter plot showing Spearman's correlation between thousand kernel weight (Y axis) and three-year mean values of free asparagine measurements (X axis).

No correlation was found between TKW and free asparagine content. These data suggested that the differences in free asparagine content among the 201 genotypes was not merely a consequence of grain filling-related processes but were probably due to a specific trait that directly influenced free asparagine accumulation.

3.3.2 Summary statistics of SNP markers and LD Decay

Among the 16,633 SNPs used for genotyping of the GDP (Mazzucotelli et al. 2017), markers with minor allele frequency < 0.05 and monomorphic markers were eliminated, resulting in 13,507 high quality informative SNPs which were used for further analysis.

In the selected population, the critical r^2 value corresponded to a distance of 4.4 Mbp (Figure 3.6). This value resulted very similar to the LD decay calculated for the *landraces* of the original collection, with the value of r^2 corresponding to a distance of 4.2 Mbp (Mazzucotelli et al. 2017). This result was expected as our population was mostly composed of *landraces* (180) with a minor contribution of the modern cultivars (21). Mazzucotelli and colleagues showed that the LD of the modern cultivars was 42.3 Mbp. Hence, the 21 modern

cultivars in our population contributed to the small LD increase compared to the LD of the landraces from Mazzucotelli et al. In addition, our data indicated that 95.5% of the genetic diversity of the original population has been maintained in the population used in this study.

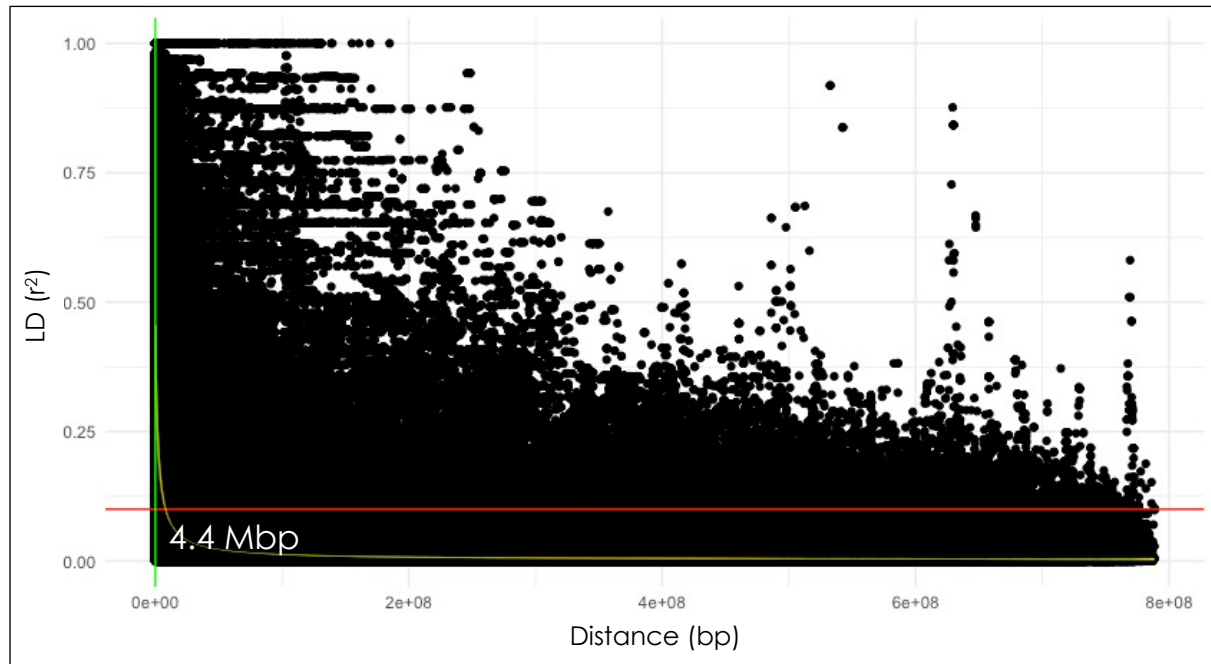


Figure 3.6: Genome wide linkage disequilibrium (LD) decay in respect to physical distance in our population.

3.3.3 Population structure analyses

The different complementary approaches such as STRUCTURE and Neighbour-Joining phylogenetic trees were employed to obtain the information on the population structure of the 201 durum wheat genotypes.

STRUCTURE analysis identified four clusters, based on BIC decreasing criterion and a K=4 was chosen taking the value of k. above which BIC values decreased (Figure 3.7, Table S3.1).

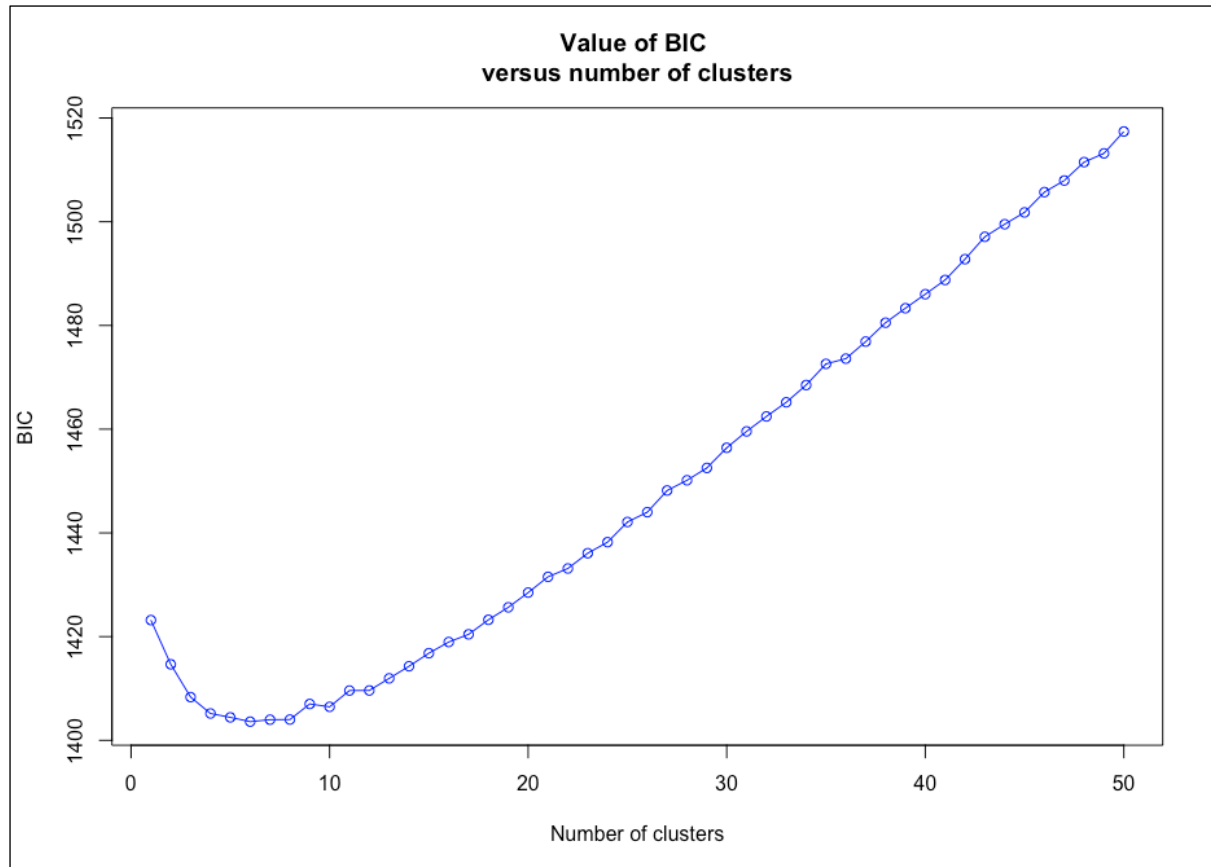


Figure 3.7: Bayesian information criterion (BIC) plotted against number of clusters.

To better understand the genetic component involved in free asparagine accumulation in our study-population, we built a neighbour-joining phylogenetic tree based on Nei's distance considering our dataset of 13,507 retained SNPs, with 1000 bootstrap replications using poppr (Kamvar et al., 2014). Then, an heatmap built using the free asparagine values obtained by the measurement of the three year field trials was associated to the phylogenetic tree (Figure 3.8).

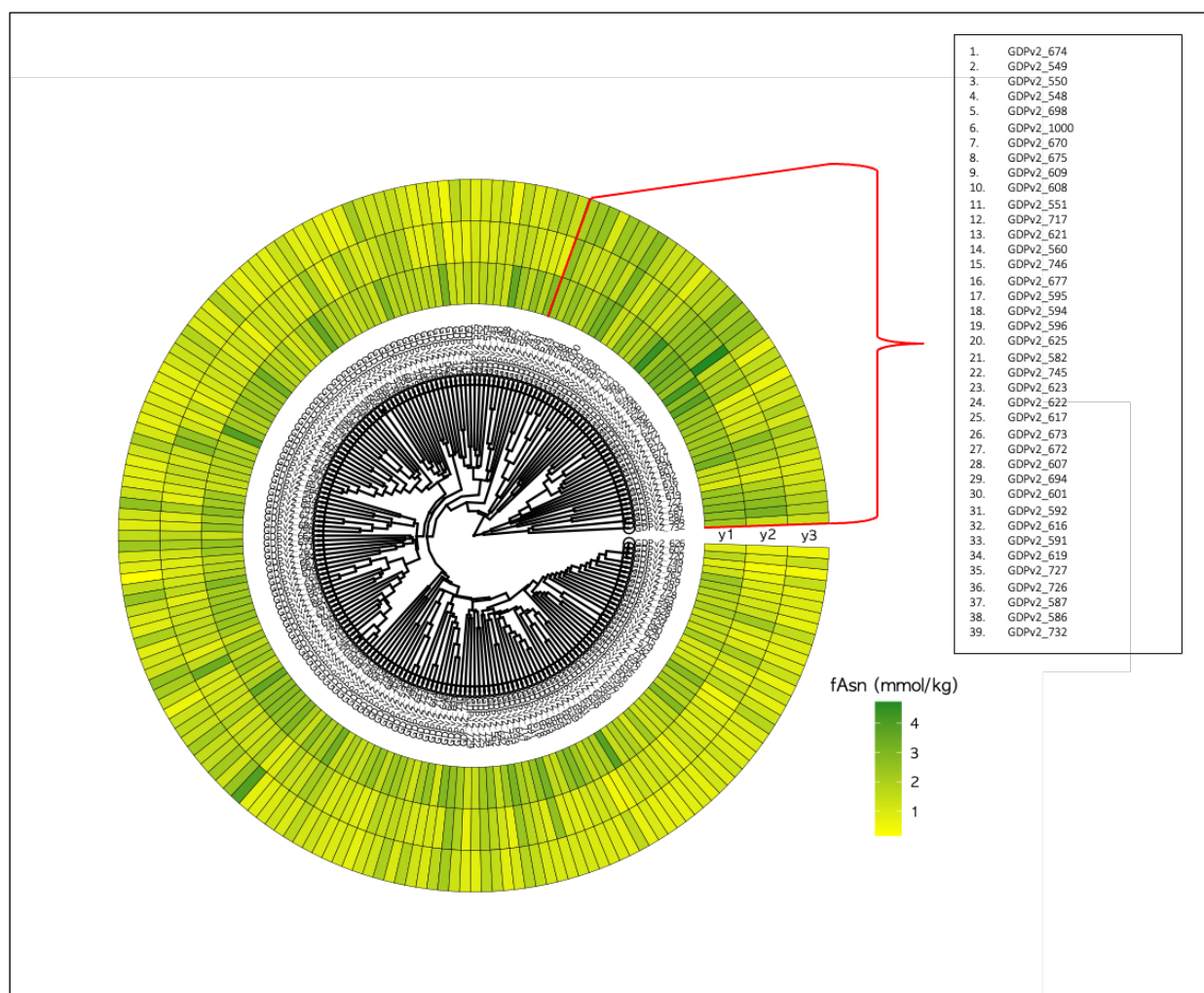


Figure 3.8: Neighbour-joining phylogenetic tree based on SNPs data with 1000 bootstrap replications. The associated heatmap shows free asparagine values of the three years. Moderate-high free asparagine clade is highlighted in red.

The analysis revealed a phylogenetic clade putatively associated to high free asparagine (highlighted in Figure 3.8). This clade includes 39 genotypes showing moderate-high levels of free asparagine content over the years. These 39 genotypes originated from 14 different countries (Tab. S3.8): among them, 34 genotypes, corresponding to about 87% of genotypes included in the clade, accumulate free asparagine with median values across year higher than the median value of the entire population (Table S3.8). This observation corroborated a genetic role in free asparagine accumulation.

3.3.4 Genome-wide association analysis

To dissect the genetic determinants controlling free asparagine accumulation in durum wheat mature seeds, we first performed a single-locus GWAS using GAPIT (Liptka et al., 2012, Tang et al., 2016) and TASSEL5 (Bradbury et al., 2007) tools. Overall, 236 and 107 SNPs were identified by GAPIT and TASSEL5, respectively, with a $-\log_{10}p > 3$ (Table S3.9 and Table S3.10).

Besides the single-locus GWAS, a mGWAS was performed using six different algorithms, since this method is known to be characterized by a higher power and accuracy than the previous used methods. Overall, 26 significant SNPs were identified by the six algorithms as associated to free asparagine accumulation at a critical threshold of $LOD > 3.0$. Among the 26 significant SNPs, six were identified by at least two different models and were considered significant QTNs in the present study (Table 3.3).

Table 3.3: Chr. chromosome, Pos. physical start position in base pairs according to the durum wheat reference genome, minor allele frequency (MAF) for which the ($\pm$) sign indicates an increasing or decreasing effect of the minor allele on the trait.

Trait	Method	Marker	Chr	Pos (bp)	QTN effect	LOD score	'-log10(P)'	r ² (%)	MAF
Free Asparagine	FASTmrEMMA	BS00108573_51	7B	545,158,872	-0.2395	5.4748	6.2893	5.574	0.2985
	FASTmrMLM	BS00108573_51	7B	545,158,872	-0.129	6.0549	6.8898	6.4688	0.2985
		GENE-4008_418	4B	609,352,811	-0.0966	3.0684	3.768	4.283	0.4478
	ISIS EM-BLASSO	BS00021957_51	7A	688,369,238	-0.1368	4.196	4.9571	6.0394	0.2239
		BS00108573_51	7B	545,158,872	-0.1451	7.745	8.6303	8.1904	0.2985
		CAP12_rep_c4638_80	6A	132,516,185	-0.1738	4.2116	4.9734	6.1079	0.1244
		GENE-4008_418	4B	609,352,811	-0.0846	3.8236	4.5662	3.287	0.4478
	mrMLM	BS00021957_51	7A	688,369,238	-0.1393	5.6979	6.5204	6.2605	0.2261
		BS00108573_51	7B	545,158,872	-0.144	7.3006	8.1738	8.0663	0.2915
		CAP12_rep_c4638_80	6A	132,516,185	-0.1649	4.6226	5.403	5.5011	0.1256
	pKWmeB	BS00062611_51	7B	697,620,145	-0.109	7.7405	8.6257	6.8778	0.3216
		Tdurum_contig67516_236	2B	405,170,690	0.0975	5.1394	5.9411	6.0183	0.4422
	pLARmeB	BS00062611_51	7B	697,620,145	-0.0744	3.8203	4.5628	1.98	0.3184
		BS00108573_51	7B	545,158,872	-0.1511	10.3097	11.2543	7.8848	0.2985
		GENE-4008_418	4B	609,352,811	-0.064	3.2535	3.9645	1.6704	0.4478
		Tdurum_contig67516_236	2B	405,170,690	0.0861	3.3431	4.0594	3.0181	0.4428

The chromosomal distribution of the six retained QTNs revealed that the B genome had four QTNs, with two of them on the same chromosome (chr. 7B), while only two QTNs were detected in genome A (Figure 3.9).

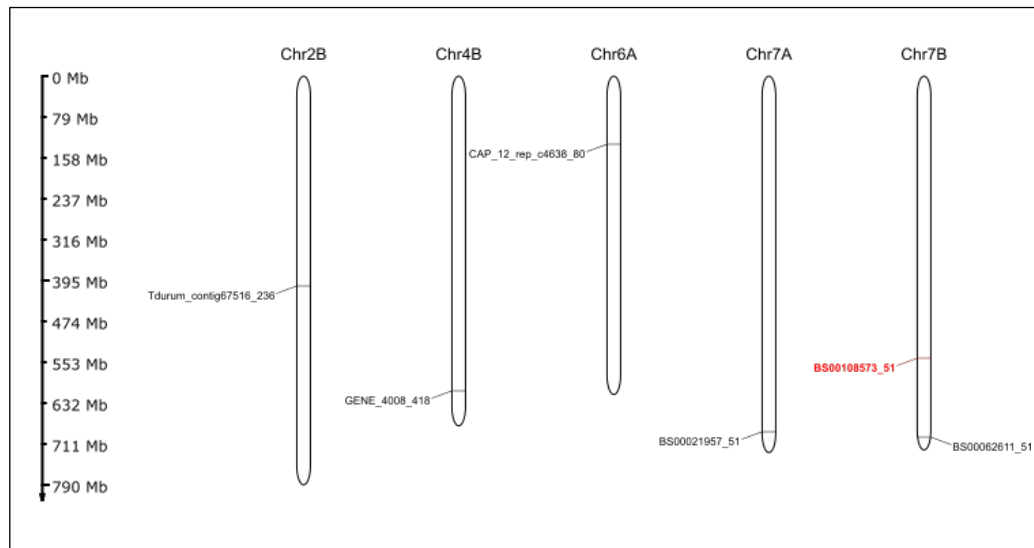


Figure 3.9: QTN positions on chromosome draw with MG2Cv2 (Chao et al., 2021). In bold/red QTNs resulted significant in all three GWAS analyses.

To identify candidate genes associated with free asparagine levels, 4.4 Mb of flanking sequences were studied for each of the retained QTNs, and the genes included in these regions are shown in Table S3.11.

The Biomart web tool (<https://www.ensembl.org/info/data/biomart/index.html>) was used to identify genes included in the map distance based on LD decay. A total of 396 genes encoding 1105 stable transcripts referred to the durum wheat reference genome (cv. Svevo) were found.

3.3.5 Evaluation of Candidate Genes within the QTN BS00108573_51

To define putative candidate genes involved in the regulation of free asparagine accumulation in the grains, we compared the results obtained with mGWAS (including six algorithms), GAPIT and TASSEL5. Among the six QTNs reporting LOD>3 in mGWAS, only the two on chromosome 7B (BS00003494_51 and BS00108573_51), showed a $-\log_{10}(\text{pvalue}) > 3$ in the other two analyses. In

particular, the QTN BS00108573_51 resulted significant in 5 on 6 algorithms included in mGWAS analysis with the highest LOD score and reported a $-\log_{10}(p) > 3$ in both TASSEL and GAPIT (6 algorithms on a total of 7) GWAS analyses (Tab. 3.4).

Table 3.4: QTN effect on phenotype for which the ($\pm$) sign indicates an increasing or decreasing effect on the trait.

	Algorithm	Tool	' $-\log_{10}(p)$ '	QTN effect
BS00108573_51	pLARmEB	mrMLM	11.2543	-0.1511
	mrMLM		8.1738	-0.144
	ISIS EM-BLASSO		8.6303	-0.1451
	FASTmrMLM		6.8898	-0.129
	FASTmrEMMA		6.2893	-0.2395
	BLINK	GAPIT	3.4662	-0.1340
	CMLM		3.4666	-0.1386
	FarmCPU		3.4663	-0.1340
	GLM		4.2315	-0.13340
	MLM		3.4666	-0.1386
	MLMM		3.6586	-0.1386
	SUPER		4.8358	NA
	TASSEL	TASSEL	4.3494	0.1313

For this reason, we searched putative candidate genes associated to free asparagine accumulation around this QTN. Fifty-four genes were present in the 4.4 Mb flanking regions (Table S3.12).

Among them, we searched for possible candidate genes that may be involved in free asparagine metabolism or transport. Free asparagine plays a central role in nitrogen storage and transport in many plant species due to its relatively high ratio of nitrogen to carbon and its unreactive nature (Lea, Sodek, Parry, Shewry, & Halford, 2007). Recently, a network describing asparagine metabolism was constructed using information available in the literature and publicly available databases (Curtis et al. 2018). This analysis underlined the complexity of this network, which consisted of 212 nodes (genes, enzymes, or molecules) and 246 edges (reactions between nodes).

The main enzymes identified as being involved in asparagine metabolism were as follows: asparagine synthetase, glutamine synthetase, glutamate dehydrogenase, ferredoxin-dependent glutamate synthase, NADH-dependent glutamate synthase, aspartate amino transferase, and glutamate decarboxylase. In addition, the involvement of specific kinases and transcription factors in the gene expression regulation was described (Curtis et al. 2018). Based on this study, among the 54 genes within the flanking regions of the QTN BS00108573_51, we searched for those genes that could be involved in transport, gene expression regulation or enzymatic activities related to amino acid metabolism. Information from gene annotation available in the genomic sequence (Maccaferri et al., 2018) and from KEGG orthology was used (Table S3.12).

Five genes putatively involved in transport of different molecules were found: TRITD7Bv1G172350, TRITD7Bv1G172420, TRITD7Bv1G171080, TRITD7Bv1G172380 and TRITD7Bv1G171560. In particular, TRITD7Bv1G171080, named *TdNPF/NRT1* (Kumar et al., 2022) codes for a putative peptide transporter and may be involved in amino acid metabolism. Its putative bread wheat ortholog *TaNPF8* shows variable expression related to nitrogen starvation/recovery growth conditions and seems to be involved in nitrate transport (Wang et al., 2020; Kumar et al., 2022).

Among the selected 54 genes, three kinases were found: TRITD7Bv1G173290, TRITD7Bv1G172950, TRITD7Bv1G171140. As reported by Curtis et al., (2018), kinase activity plays a key role in the expression regulation of asparagine synthetase genes. A role of one of these kinases in the regulation of genes involved in the described network is plausible. Moreover, two transcription factors were present, a *TCP* (TRITD7Bv1G172970) and a *MYB* (TRITD7Bv1G173450). In *Arabidopsis*, *TCP* transcription factors were described for their involvement in N metabolism and in plant responses to nitrate availability (Guan et al. 2017). Interestingly, a rice *MYB* transcription factor was described for its role in regulating amino acid metabolism (El-kereamy et al., 2012), suggesting that also the durum wheat *MYB* gene TRITD7Bv1G173450 may

be involved in amino acid metabolism regulation. Pearce and collaborators (2015) performed a transcriptomic analysis on different tissues (inner pericarp, outer pericarp and endosperm) of developing grain of bread wheat and found that the orthologue MYB gene TraesCS7B02G315800 was expressed in outer pericarp and not in other seed layers. Consistently, free asparagine accumulation reaches the highest levels in the bran fractions and in particular the aleurone layer (Shewry et al., 2009). Moreover, an involvement of the bread wheat MYB gene TraesCS7B02G315800 in gibberellin pathway was reported (Pearce et al., 2015). The role of gibberellins in regulating nitrogen uptake and other physiological traits in maize in response to nitrogen availability was described (Wang et al. 2022).

Among the genes coding for enzymes with activity related to amino acid metabolism, two L-lactate dehydrogenases (TRITD7Bv1G172590 and TRITD7Bv1G172680), that are involved in cysteine and methionine metabolism, and an oligosaccharyltransferase, that could be involved in the binding between asparagine and glycans (Curtis et al. 2018), were also present.

These genes may be putative candidates involved in the regulation of asparagine metabolism. Unfortunately, literature information on asparagine metabolism in wheat grain, as well as functional characterization of durum wheat genes, are very few and further analysis are needed to evaluate a possible involvement of these genes in the regulation of free asparagine amount in the seeds.

3.4. Conclusion

In this work, we performed a mGWAS related to free asparagine content in durum wheat seeds. We performed a three-year phenotypic analysis on 201 durum wheat genotypes, mostly landraces, which increased the genetic variability of the selected panel. The analysis showed the presence of significant variability for free asparagine content in the chosen population. The association study identified six QTNs and related genomic regions on chromosomes 6A, 7A, 2B, 4B, 7B associated to free asparagine content. To our

knowledge, this is the first GWAS on durum wheat for this trait. The obtained results will be very useful to identify good candidates for future functional characterization studies aimed to dissect the genetic architecture that regulates free asparagine accumulation in wheat grain, in response to crop management practices, environmental conditions and biotic and abiotic stresses.

3.5 Funding

This work was partially funded by the European Regional Development Fund under the ROP of the Lombardy Region ERDF 2014–2020—Axis I “Strengthen technological research, development and innovation”—Action 1.b.1.3 “Support for co-operative R&D activities to develop new sustainable technologies, products and services”—Call Hub.

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3.7 Supplementary material

Table S3.1: List of genotypes

	FINAL accession code	Accession name	Plant species	Accession history	Country of collection / development	Clusters in Tafuri et al.
1	GDPv2_069	MERIDIANO	T. durum	cv	ITA	2
2	GDPv2_091	CAPPELLI	T. durum	landrace	ITA	4
3	GDPv2_1000	DWGIGSSALATFINA020	T. durum	landrace	IND	1
4	GDPv2_105	SVEVO	T. durum	cv	ITA	2
5	GDPv2_282	Simeto	T. durum	cv	ITA	4
6	GDPv2_347	Aureo	T. durum	cv	ITA	2
7	GDPv2_379	Vendetta	T. durum	cv	ITA	4
8	GDPv2_548	ENGB-Sohag-7	T. durum	landrace	EGY	1
9	GDPv2_549	ENGB-Qena-10	T. durum	landrace	EGY	1
10	GDPv2_550	ENGB-Sohag-12	T. durum	landrace	EGY	1
11	GDPv2_551	ENGB-USDA-13	T. durum	landrace	EGY	1
12	GDPv2_552	Kilinto	T. durum	cv	ETH	2
13	GDPv2_554	Robe	T. durum	cv	ETH	2
14	GDPv2_555	Boohai	T. durum	cv	ETH	4
15	GDPv2_556	Jordan_Coll_86_172	T. durum	landrace	JOR	4
16	GDPv2_557	Jordan_Coll_Landrace_164	T. durum	landrace	JOR	2
17	GDPv2_558	Karabilcik_23-a	T. durum	landrace	TUR	2
18	GDPv2_559	Karakilcik_113-b	T. durum	landrace	TUR	4
19	GDPv2_560	Karisik_242/2	T. durum	landrace	TUR	1
20	GDPv2_561	Kirmize_173-b	T. durum	landrace	TUR	2
21	GDPv2_562	Kirmizi_54-C	T. durum	landrace	TUR	4
22	GDPv2_563	Kishk	T. durum	landrace	TUR	4
23	GDPv2_564	Lebrija	T. durum	landrace	ESP	4
24	GDPv2_565	Bonzo	T. durum	landrace	ESP	2
25	GDPv2_566	Abu_Fashit_IRTA	T. durum	landrace	ISR	1
26	GDPv2_567	Juljolith	T. durum	landrace	ISR	1
27	GDPv2_569	Bidi17	T. durum	landrace	TUN	4
28	GDPv2_570	Almaz	T. durum	cv	KAZ	2
29	GDPv2_571	Kostanayskaya_12	T. durum	cv	KAZ	2
30	GDPv2_572	Kostanayskaya_52	T. durum	cv	KAZ	2
31	GDPv2_573	Kharkovskaya_46	T. durum	landrace	KAZ	2
32	GDPv2_574	Saratovskaya_31	T. durum	landrace	KAZ	2
33	GDPv2_575	Altaiski_yanvar	T. durum	cv	KAZ	2
34	GDPv2_576	ordeiforme_254	T. durum	cv	KAZ	2
35	GDPv2_577	Karabalykskaya_chernokolosaya_20	T. durum	cv	KAZ	2

36	GDPv2_578	Omski_rubin	T. durum	cv	KAZ	2
37	GDPv2_579	DWGIGSSALATFINA035	T. durum	landrace	ITA	4
38	GDPv2_580	DWGIGSSALATFINA036	T. durum	landrace	SYR	1
39	GDPv2_581	DWGIGSSALATFINA053	T. durum	landrace	JOR	4
40	GDPv2_582	DWGIGSSALATFINA057	T. durum	landrace	ESP	3
41	GDPv2_583	DW-SR-FIGS027	T. durum	landrace	ETH	1
42	GDPv2_584	DW-SR-FIGS049	T. durum	landrace	AFG	1
43	GDPv2_585	DW-SR-FIGS063	T. durum	landrace	IRQ	1
44	GDPv2_586	DW-SR-FIGS082	T. durum	landrace	GRC	3
45	GDPv2_587	DW-SR-FIGS089	T. durum	landrace	AFG	3
46	GDPv2_588	DW-SR-FIGS096	T. durum	landrace	DZA	2
47	GDPv2_589	DW-SR-FIGS103	T. durum	landrace	DZA	4
48	GDPv2_590	DW-SR-FIGS119	T. durum	landrace	MAR	4
49	GDPv2_591	FIGSDRYWET001	T. durum	landrace	OMN	1
50	GDPv2_592	FIGSDRYWET003	T. durum	landrace	ETH	3
51	GDPv2_593	FIGSDRYWET007	T. durum	landrace	RUS	4
52	GDPv2_594	FIGSDRYWET028	T. durum	landrace	ETH	3
53	GDPv2_595	FIGSDRYWET029	T. durum	landrace	ETH	3
54	GDPv2_596	FIGSDRYWET030	T. durum	landrace	ETH	3
55	GDPv2_597	FIGSDRYWET033	T. durum	landrace	TUR	2
56	GDPv2_598	FIGSDRYWET038	T. durum	landrace	ESP	4
57	GDPv2_599	FIGSDRYWET051	T. durum	landrace	AFG	2
58	GDPv2_600	FIGSDRYWET053	T. durum	landrace	AFG	2
59	GDPv2_601	FIGSDRYWET058	T. durum	landrace	ETH	1
60	GDPv2_602	FIGSDRYWET060	T. durum	landrace	ETH	4
61	GDPv2_603	FIGSDRYWET078	T. durum	landrace	AFG	4
62	GDPv2_604	FIGSDRYWET098	T. durum	landrace	JOR	1
63	GDPv2_605	FIGSDRYWET107	T. durum	landrace	KAZ	2
64	GDPv2_606	FIGSDRYWET109	T. durum	landrace	TUR	4
65	GDPv2_607	FIGSDRYWET110	T. durum	landrace	YEM	3
66	GDPv2_608	FIGSDRYWET115	T. durum	landrace	YEM	1
67	GDPv2_609	FIGSDRYWET120	T. durum	landrace	YEM	1
68	GDPv2_610	FIGSDRYWET130	T. durum	landrace	SYR	1
69	GDPv2_611	FIGSDRYWET143	T. durum	landrace	IRN	1
70	GDPv2_612	FIGSDRYWET144	T. durum	landrace	IRN	1
71	GDPv2_613	FIGSDRYWET147	T. durum	landrace	ARM	2
72	GDPv2_614	FIGSDRYWET163	T. durum	landrace	RUS	1
73	GDPv2_615	FIGSDRYWET174	T. durum	landrace	ARM	2
74	GDPv2_616	FIGSDWHOTCLD005	T. durum	landrace	OMN	1
75	GDPv2_617	FIGSDWHOTCLD009	T. durum	landrace	ETH	3
76	GDPv2_618	FIGSDWHOTCLD021	T. durum	landrace	AZE	4
77	GDPv2_619	FIGSDWHOTCLD051	T. durum	landrace	SAU	1

78	GDPv2_620	FIGSDWHOTCLD063	T. durum	landrace	ESP	4
79	GDPv2_621	FIGSDWHOTCLD090	T. durum	landrace	YUG	1
80	GDPv2_622	FIGSDWHOTCLD093	T. durum	landrace	ETH	3
81	GDPv2_623	FIGSDWHOTCLD107	T. durum	landrace	ETH	3
82	GDPv2_624	FIGSDWHOTCLD133	T. durum	landrace	ETH	4
83	GDPv2_625	FIGSDWHOTCLD139	T. durum	landrace	ETH	3
84	GDPv2_626	FIGSDWHOTCLD144	T. durum	landrace	ITA	4
85	GDPv2_627	FIGSDWHOTCLD147	T. durum	landrace	ITA	1
86	GDPv2_628	FIGSDWHOTCLD149	T. durum	landrace	ITA	4
87	GDPv2_629	FIGSDWHOTCLD166	T. durum	landrace	GRC	4
88	GDPv2_630	FIGSDWHOTCLD168	T. durum	landrace	ETH	4
89	GDPv2_631	FIGSDWHOTCLD182	T. durum	landrace	ITA	4
90	GDPv2_632	FIGSDWHOTCLD188	T. durum	landrace	PAK	4
91	GDPv2_633	Baladi	T. durum	landrace	SYR	1
92	GDPv2_634	Biadi	T. durum	landrace	SYR	1
93	GDPv2_635	Al_Kaf	T. durum	landrace	TUN	4
94	GDPv2_636	Safaqis[Sfax]	T. durum	landrace	TUN	4
95	GDPv2_637	Susah	T. durum	landrace	TUN	4
96	GDPv2_638	Chelf	T. durum	landrace	DZA	4
97	GDPv2_639	SSD_002	T. durum	landrace	DZA	4
98	GDPv2_640	SSD_003	T. durum	landrace	DZA	2
99	GDPv2_641	SSD_004	T. durum	landrace	ESP	4
100	GDPv2_642	SSD_006	T. durum	landrace	DZA	4
101	GDPv2_643	SSD_007	T. durum	landrace	PRT	1
102	GDPv2_644	SSD_011	T. durum	landrace	TUN	4
103	GDPv2_645	SSD_017	T. durum	landrace	TUN	4
104	GDPv2_646	SSD_027	T. durum	landrace	TUN	4
105	GDPv2_647	SSD_028	T. durum	landrace	TUN	4
106	GDPv2_648	SSD_041	T. durum	landrace	TUN	2
107	GDPv2_649	SSD_043	T. durum	landrace	TUN	4
108	GDPv2_650	SSD_044	T. durum	landrace	TUN	2
109	GDPv2_651	SSD_047	T. durum	landrace	UKR	2
110	GDPv2_652	SSD_048	T. durum	landrace	UKR	2
111	GDPv2_653	SSD_049	T. durum	landrace	RUS	1
112	GDPv2_654	SSD_052	T. durum	landrace	EGY	4
113	GDPv2_655	SSD_064	T. durum	landrace	MAR	4
114	GDPv2_658	SSD_067	T. durum	landrace	MAR	4
115	GDPv2_659	SSD_069	T. durum	landrace	MAR	4
116	GDPv2_660	SSD_092	T. durum	landrace	USA	1
117	GDPv2_661	SSD_107	T. durum	landrace	IRQ	1
118	GDPv2_662	SSD_109	T. durum	landrace	IRQ	1
119	GDPv2_663	SSD_111	T. durum	landrace	IRQ	1

120	GDPv2_664	SSD_112	T. durum	landrace	IRQ	1
121	GDPv2_666	SSD_115	T. durum	landrace	IRQ	2
122	GDPv2_667	SSD_118	T. durum	landrace	SRB	2
123	GDPv2_668	SSD_128	T. durum	landrace	USA	2
124	GDPv2_669	SSD_137	T. durum	landrace	TUR	4
125	GDPv2_670	SSD_142	T. durum	landrace	PRT	1
126	GDPv2_671	SSD_155	T. durum	landrace	IND	4
127	GDPv2_672	SSD_157	T. durum	landrace	TUN	3
128	GDPv2_673	SSD_158	T. durum	landrace	ETH	3
129	GDPv2_674	SSD_162	T. durum	landrace	SAU	1
130	GDPv2_675	SSD_173	T. durum	landrace	EGY	1
131	GDPv2_677	SSD_182	T. durum	landrace	IND	1
132	GDPv2_678	SSD_188	T. durum	landrace	SYR	1
133	GDPv2_679	SSD_191	T. durum	landrace	SYR	1
134	GDPv2_680	SSD_195	T. durum	landrace	SAU	4
135	GDPv2_681	SSD_239	T. durum	landrace	ETH	1
136	GDPv2_683	SSD_244	T. durum	landrace	ETH	4
137	GDPv2_685	SSD_253	T. durum	landrace	CYP	4
138	GDPv2_686	SSD_255	T. durum	landrace	AFG	2
139	GDPv2_687	SSD_256	T. durum	landrace	IRN	1
140	GDPv2_688	SSD_262	T. durum	landrace	IRN	2
141	GDPv2_689	SSD_264	T. durum	landrace	ITA	1
142	GDPv2_692	SSD_278	T. durum	landrace	BGR	1
143	GDPv2_693	SSD_292	T. durum	landrace	TUN	4
144	GDPv2_694	SSD_294	T. durum	landrace	TUN	3
145	GDPv2_695	SSD_302	T. durum	landrace	TUN	4
146	GDPv2_696	SSD_303	T. durum	landrace	TUN	4
147	GDPv2_697	SSD_315	T. durum	landrace	LBY	4
148	GDPv2_698	SSD_322	T. durum	landrace	TUR	1
149	GDPv2_699	SSD_325	T. durum	landrace	SYR	1
150	GDPv2_701	SSD_336	T. durum	landrace	IRQ	4
151	GDPv2_702	SSD_348	T. durum	landrace	IRN	1
152	GDPv2_704	SSD_407	T. durum	landrace	GRC	2
153	GDPv2_705	SSD_411	T. durum	landrace	GRC	1
154	GDPv2_706	SSD_412	T. durum	landrace	GRC	4
155	GDPv2_707	SSD_415	T. durum	landrace	GRC	1
156	GDPv2_708	SSD_416	T. durum	landrace	GRC	1
157	GDPv2_709	SSD_431	T. durum	landrace	GRC	1
158	GDPv2_710	SSD_443	T. durum	landrace	GRC	1
159	GDPv2_711	SSD_451	T. durum	landrace	IRQ	1
160	GDPv2_714	SSD_470	T. durum	landrace	ESP	4
161	GDPv2_715	SSD_477	T. durum	landrace	ITA	4

162	GDPv2_716	SSD_482	T. durum	landrace	ITA	4
163	GDPv2_717	SSD_483	T. durum	landrace	GRC	1
164	GDPv2_719	SSD_491	T. durum	landrace	GRC	1
165	GDPv2_720	SSD_500	T. durum	landrace	ITA	4
166	GDPv2_721	SSD_531	T. durum	landrace	ITA	1
167	GDPv2_722	BIANCUCCIA	T. durum	landrace	ITA	4
168	GDPv2_723	BIVONA	T. turgidum	landrace	ITA	4
169	GDPv2_724	BUFALA_Rossa_Lunga	T. turgidum	landrace	ITA	4
170	GDPv2_725	CASTIGLIONE	T. durum	landrace	ITA	4
171	GDPv2_726	CICIREDDA	T. turgidum	landrace	ITA	3
172	GDPv2_727	CURCITTA	T. durum	landrace	ITA	3
173	GDPv2_728	FARRO_LUNGO	T. durum	landrace	ITA	4
174	GDPv2_729	GIOIA	T. durum	landrace	ITA	4
175	GDPv2_730	INGLESA	T. durum	landrace	ITA	4
176	GDPv2_731	LINA	T. durum	landrace	ITA	4
177	GDPv2_732	PAOLA	T. durum	landrace	ITA	3
178	GDPv2_734	SAMMARTINARA	T. durum	landrace	ITA	4
179	GDPv2_736	CANU_ARDESU	T. durum	landrace	ITA	4
180	GDPv2_737	CANU_BASCIU	T. durum	landrace	ITA	4
181	GDPv2_738	LEUCURUM-3	T. durum	cv	UZB	1
182	GDPv2_739	MARVARID	T. durum	cv	UZB	2
183	GDPv2_740	YOQUT-2014	T. durum	cv	UZB	2
184	GDPv2_741	MINGCHINOR	T. durum	cv	UZB	2
185	GDPv2_742	ISTIQBOLLI	T. durum	cv	UZB	2
186	GDPv2_743	MAKUZ-3	T. durum	cv	UZB	2
187	GDPv2_745	PI480352	T. durum	landrace	ETH	3
188	GDPv2_746	PI204020_ Vermelho de Barba Preta	T. durum	landrace	PRT	1
189	GDPv2_747	Aziziah	T. durum	landrace	ITA	1
190	GDPv2_748	Kyperounda	T. durum	landrace	MAR	4
191	GDPv2_749	Taganrog	T. durum	landrace	ITA	4
192	GDPv2_750	Timilia	T. durum	landrace	ITA	1
193	GDPv2_752	377b	T. durum	landrace	ETH	4
194	GDPv2_754	Akbasak	T. durum	landrace	TUR	1
195	GDPv2_755	Azulejo	T. durum	landrace	ESP	4
196	GDPv2_757	Hordeiforme_27	T. durum	landrace	RUS	2
197	GDPv2_758	Abyssinicum	T. durum	landrace	UK	4
198	GDPv2_760	T-2	T. durum	landrace	PAK	1
199	GDPv2_761	IQ_118	T. durum	landrace	IRQ	1
200	GDPv2_762	Mamhoudi	T. durum	landrace	TUN	4
201	GDPv2_763	IWA8609000	T. turanicum	landrace	IRN	1

Table S3.2: Fertilization inputs.

Year	Treatment date	Fertilizer	Quantity
2019/2020	pre-sowing	N	30kg/ha
		P ₂ O ₅	30kg/ha
		K ₂ O	30kg/ha
	11/03/2020	Nitrate N 27%	150 kg/ha
	28/04/2020	Nitrate N 27%	100 kg/ha
2020/2021	03/03/2021	Nitrate N 27%	150 kg/ha
	03/05/2021	Nitrate N 27%	100 kg/ha
2021/2022	01/03/2022	N 29%	150 kg/ha
	20/04/2022	Nitrate N 27%	100 kg/ha

Table S3.3.1: Weather data first year field trial.

2019/2020				
decade	month	Tmin (°C)	Tmax (°C)	Rain (mm)
I	October	9.28	20.54	21.10
II	October	11.55	19.45	77.20
III	October	12.94	18.98	11.30
I	November	6.58	13.10	79.00
II	November	4.25	10.12	291.40
III	November	6.55	11.86	23.00
I	December	1.16	9.01	17.90
II	December	2.37	8.03	105.70
III	December	1.00	10.40	54.50
I	January	-2.49	7.06	0.40
II	January	-0.63	8.02	10.20
III	January	-0.24	9.86	2.40
I	February	0.44	11.58	0.60
II	February	0.32	14.80	0.00
III	February	0.85	14.38	0.00
I	March	1.20	11.18	42.20
II	March	2.54	17.13	0.00
III	March	1.73	12.97	7.60
I	April	1.07	18.95	0.00
II	April	5.90	22.18	20.20
III	April	8.53	21.67	11.00
I	May	11.28	24.81	1.20
II	May	15.29	23.29	16.20
III	May	15.63	26.57	3.40
I	June	15.63	25.07	39.40
II	June	16.25	26.14	36.80
III	June	20.33	31.61	0.00

Table S3.3.2: Weather data second year field trial.

2020/2021				
decade	month	Tmin (°C)	Tmax (°C)	Rain (mm)
I	October	12.47	21.4	n.a.
II	October	8.27	16.4	n.a.
III	October	9.85	16.7	n.a.
I	November	9.40	16.0	n.a.
II	November	8.05	12.7	n.a.
III	November	2.00	10.2	n.a.
I	December	2.25	5.5	n.a.
II	December	3.06	7.9	n.a.
III	December	1.35	6.4	n.a.
I	January	0.90	4.4	n.a.
II	January	-1.42	5.3	n.a.
III	January	1.92	8.7	n.a.
I	February	5.69	9.3	n.a.
II	February	1.88	9.4	n.a.
III	February	5.85	17.0	n.a.
I	March	3.91	14.5	n.a.
II	March	4.18	15.5	n.a.
III	March	6.39	26.0	n.a.
I	April	6.63	18.7	n.a.
II	April	6.12	15.5	n.a.
III	April	10.98	23.3	n.a.
I	May	11.98	22.7	n.a.
II	May	12.05	15.5	n.a.
III	May	13.44	26.0	n.a.
I	June	17.33	28.5	n.a.
II	June	20.36	15.5	n.a.
III	June	19.96	25.5	n.a.

Table S3.3.3: Weather data third year field trail.

2021/2022				
decade	month	Tmin (°C)	Tmax (°C)	Rain (mm)
I	October	10.95	20.06	20.20
II	October	3.68	18.38	0.00
III	October	4.48	16.34	2.00
I	November	4.57	12.09	53.80
II	November	6.78	11.17	65.40
III	November	0.99	8.33	25.20
I	December	-2.63	5.22	16.60
II	December	-5.57	3.42	4.60
III	December	0.40	4.68	16.80
I	January	-1.41	4.57	24.00
II	January	-4.44	8.38	5.80
III	January	-3.15	6.29	0.80
I	February	-1.53	12.48	0.20
II	February	-1.21	9.23	26.00
III	February	-1.68	14.43	0.00
I	March	-3.73	11.48	0.00
II	March	0.79	12.93	0.00
III	March	0.48	17.55	14.20
I	April	2.13	16.42	19.80
II	April	2.74	19.68	0.00
III	April	5.54	18.48	14.20
I	May	7.98	20.53	30.40
II	May	11.41	28.87	0.00
III	May	13.64	26.69	17.20
I	June	14.07	28.19	5.00
II	June	16.30	32.77	29.80
III	June	16.28	31.68	23.00

Table S3.4: Soil analysis results.

		2019/2020			2020/2021			2020/2021		
		Value	±	Unit	Value	±	Unit	Value	±	Unit
N		0.12	0.01	%	1.57	0.01	%	1.66	0.01	%
C		1.15	0.06	%	1.22	0.06	%	1.25	0.06	%
organic matter		1.98	0.1	%	2.11	0.1	%	2.16	0.1	%
C/N		9.6	-	-	7.8	-	-	7.5	-	-
texture	Clay	36	-	%	30	-	%	32.5	-	%
	silt	17	-	%	26	-	%	52.2	-	%
	sand	47	-	%	45	-	%	15.3	-	%
CaCo3 total		11.8	0.6	%	25	0.6	%	11	0.6	%
CaCO3 active		3.1	0.2	%	7.2	0.2	%	2.1	0.2	%
P2O5		35	4	mg/Kg	42	4	mg/Kg	41	4	mg/Kg
Ca		9527	476	mg/Kg	5702	476	mg/Kg	5832	476	mg/Kg
Mg		505	25	mg/Kg	472	25	mg/Kg	1050	25	mg/Kg
Na		186	9	mg/Kg	112	9	mg/Kg	48	9	mg/Kg

K	239	12	mg/Kg	170	12	mg/Kg	191	12	mg/Kg
pH at 20 °C	7.8	0.1	Unità pH	8	0.1	Unità pH	7.9	0.1	Unità pH
Electrical conductivity at 25°C	1.1	0.03	dS/m	1.62	0.03	dS/m	1.53	0.03	dS/m

Table S3.5: Free asparagine measurements.

FINAL accession code	Name	Species	History	fAsn (mmol/kg)	fAsn (mmol/kg)	fAsn (mmol/kg)
				2020	2021	2022
GDPv2_091	Cappelli	T. durum	landrace	1.66	1.38	0.74
GDPv2_548	ENGB-Sohag-7	T. durum	landrace	2.05	1.48	1.05
GDPv2_549	ENGB-Qena-10	T. durum	landrace	1.93	1.84	2.37
GDPv2_550	ENGB-Sohag-12	T. durum	landrace	2.63	1.37	2.54
GDPv2_551	ENGB-USDA-13	T. durum	landrace	2.81	2.31	1.45
GDPv2_556	Jordan_Coll_86_172	T. durum	landrace	1.39	0.68	0.73
GDPv2_557	Jordan_Coll_Landrace_164	T. durum	landrace	1.69	1.13	1.08
GDPv2_558	Karabilcik_23-a	T. durum	landrace	1.90	1.47	0.61
GDPv2_559	Karakilcik_113-b	T. durum	landrace	1.95	2.15	0.66
GDPv2_560	Karisik_242/2	T. durum	landrace	3.01	1.86	1.79
GDPv2_561	Kirmize_173-b	T. durum	landrace	1.72	1.12	1.00
GDPv2_562	Kirmizi_54-C	T. durum	landrace	1.50	1.16	0.80
GDPv2_563	Kishk	T. durum	landrace	1.36	1.09	0.98
GDPv2_564	Lebrija	T. durum	landrace	2.11	2.06	1.37
GDPv2_565	Bonzo	T. durum	landrace	2.65	1.03	1.76
GDPv2_566	Abu_Fashit_Conx	T. durum	landrace	1.73	1.37	2.03
GDPv2_567	Juljolith	T. durum	landrace	1.82	1.19	1.20
GDPv2_569	Bidi17	T. durum	landrace	1.80	1.16	0.84
GDPv2_573	Kharkovskaya_46	T. durum	landrace	2.04	0.95	0.75
GDPv2_574	Saratovskaya_31	T. durum	landrace	2.38	1.16	1.45
GDPv2_1000	DWGIGSSALATFINA020	T. durum	landrace	2.67	2.30	2.21
GDPv2_579	DWGIGSSALATFINA035	T. durum	landrace	2.81	0.50	1.81
GDPv2_580	DWGIGSSALATFINA036	T. durum	landrace	2.83	1.58	1.56
GDPv2_581	DWGIGSSALATFINA053	T. durum	landrace	3.82	1.09	1.01
GDPv2_582	DWGIGSSALATFINA057	T. durum	landrace	4.10	1.57	1.63
GDPv2_583	DW-SR-FIGS027	T. durum	landrace	2.78	1.16	1.52
GDPv2_584	DW-SR-FIGS049	T. durum	landrace	1.76	1.00	0.16
GDPv2_585	DW-SR-FIGS063	T. durum	landrace	1.97	1.86	1.35
GDPv2_586	DW-SR-FIGS082	T. durum	landrace	2.63	3.12	1.92
GDPv2_587	DW-SR-FIGS089	T. durum	landrace	2.82	2.79	1.85
GDPv2_588	DW-SR-FIGS096	T. durum	landrace	3.63	1.61	1.66
GDPv2_589	DW-SR-FIGS103	T. durum	landrace	1.13	1.12	0.83
GDPv2_590	DW-SR-FIGS119	T. durum	landrace	2.18	1.06	0.95
GDPv2_591	FIGSDRYWET001	T. durum	landrace	1.44	1.19	1.06
GDPv2_592	FIGSDRYWET003	T. durum	landrace	3.22	2.10	1.54
GDPv2_593	FIGSDRYWET007	T. durum	landrace	2.10	1.71	1.52
GDPv2_594	FIGSDRYWET028	T. durum	landrace	3.28	2.80	2.11
GDPv2_595	FIGSDRYWET029	T. durum	landrace	3.29	2.74	3.00
GDPv2_596	FIGSDRYWET030	T. durum	landrace	3.07	2.34	2.66
GDPv2_597	FIGSDRYWET033	T. durum	landrace	2.56	1.16	1.26
GDPv2_598	FIGSDRYWET038	T. durum	landrace	1.65	0.74	0.80
GDPv2_599	FIGSDRYWET051	T. durum	landrace	2.17	1.45	1.08
GDPv2_600	FIGSDRYWET053	T. durum	landrace	1.13	1.34	1.22
GDPv2_601	FIGSDRYWET058	T. durum	landrace	2.64	2.82	2.14
GDPv2_602	FIGSDRYWET060	T. durum	landrace	1.71	0.88	1.39
GDPv2_603	FIGSDRYWET078	T. durum	landrace	2.09	0.88	3.95
GDPv2_604	FIGSDRYWET098	T. durum	landrace	2.48	1.45	1.30

GDPv2_605	FIGSDRYWET107	T. durum	landrace	2.18	1.30	1.34
GDPv2_606	FIGSDRYWET109	T. durum	landrace	1.75	1.45	0.87
GDPv2_607	FIGSDRYWET110	T. durum	landrace	2.31	1.23	2.00
GDPv2_608	FIGSDRYWET115	T. durum	landrace	1.50	1.11	1.35
GDPv2_609	FIGSDRYWET120	T. durum	landrace	1.48	1.69	1.86
GDPv2_610	FIGSDRYWET130	T. durum	landrace	3.58	1.28	1.37
GDPv2_611	FIGSDRYWET143	T. durum	landrace	2.18	1.48	1.17
GDPv2_612	FIGSDRYWET144	T. durum	landrace	2.48	1.34	0.87
GDPv2_613	FIGSDRYWET147	T. durum	landrace	2.15	1.37	1.01
GDPv2_614	FIGSDRYWET163	T. durum	landrace	1.50	1.56	0.75
GDPv2_615	FIGSDRYWET174	T. durum	landrace	1.43	0.63	0.82
GDPv2_616	FIGSDWHOTCLD005	T. durum	landrace	1.42	1.96	1.68
GDPv2_617	FIGSDWHOTCLD009	T. durum	landrace	2.37	0.76	0.53
GDPv2_618	FIGSDWHOTCLD021	T. durum	landrace	1.62	1.01	1.11
GDPv2_619	FIGSDWHOTCLD051	T. durum	landrace	0.99	0.97	1.01
GDPv2_620	FIGSDWHOTCLD063	T. durum	landrace	2.72	1.83	0.85
GDPv2_621	FIGSDWHOTCLD090	T. durum	landrace	2.00	1.27	0.82
GDPv2_622	FIGSDWHOTCLD093	T. durum	landrace	2.64	2.39	1.61
GDPv2_623	FIGSDWHOTCLD107	T. durum	landrace	3.78	1.84	1.58
GDPv2_624	FIGSDWHOTCLD133	T. durum	landrace	1.11	1.36	0.67
GDPv2_625	FIGSDWHOTCLD139	T. durum	landrace	2.49	4.73	2.52
GDPv2_626	FIGSDWHOTCLD144	T. durum	landrace	0.91	0.66	0.50
GDPv2_627	FIGSDWHOTCLD147	T. durum	landrace	2.22	0.77	1.39
GDPv2_628	FIGSDWHOTCLD149	T. durum	landrace	1.37	1.13	0.89
GDPv2_629	FIGSDWHOTCLD166	T. durum	landrace	1.26	1.41	0.75
GDPv2_630	FIGSDWHOTCLD168	T. durum	landrace	2.39	0.99	0.83
GDPv2_631	FIGSDWHOTCLD182	T. durum	landrace	2.38	1.74	1.15
GDPv2_632	FIGSDWHOTCLD188	T. durum	landrace	2.89	0.93	0.97
GDPv2_633	Baladi	T. durum	landrace	3.00	2.04	2.45
GDPv2_634	Biadi	T. durum	landrace	2.60	1.26	2.22
GDPv2_635	Al_Kaf	T. durum	landrace	1.65	1.09	1.29
GDPv2_636	Safaqis[Sfax]	T. durum	landrace	1.43	1.16	1.19
GDPv2_637	Susah	T. durum	landrace	1.48	1.39	0.73
GDPv2_638	Chelf	T. durum	landrace	1.93	1.07	0.80
GDPv2_639	SSD_002	T. durum	landrace	2.02	0.52	1.63
GDPv2_640	SSD_003	T. durum	landrace	3.88	0.84	0.97
GDPv2_641	SSD_004	T. durum	landrace	3.18	2.59	1.05
GDPv2_642	SSD_006	T. durum	landrace	2.30	0.70	1.05
GDPv2_643	SSD_007	T. durum	landrace	2.26	1.36	1.30
GDPv2_644	SSD_011	T. durum	landrace	1.26	1.39	1.05
GDPv2_645	SSD_017	T. durum	landrace	1.17	1.15	1.02
GDPv2_646	SSD_027	T. durum	landrace	2.57	1.06	1.22
GDPv2_647	SSD_028	T. durum	landrace	1.84	1.31	1.11
GDPv2_648	SSD_041	T. durum	landrace	1.20	1.13	0.68
GDPv2_649	SSD_043	T. durum	landrace	1.92	1.36	0.72
GDPv2_650	SSD_044	T. durum	landrace	1.82	1.17	0.86
GDPv2_651	SSD_047	T. durum	landrace	2.20	2.92	1.06
GDPv2_652	SSD_048	T. durum	landrace	2.10	1.31	1.20
GDPv2_653	SSD_049	T. durum	landrace	1.33	1.32	0.81
GDPv2_654	SSD_052	T. durum	landrace	2.28	1.25	0.97
GDPv2_655	SSD_064	T. durum	landrace	1.66	1.30	0.78
GDPv2_658	SSD_067	T. durum	landrace	1.51	0.97	1.11
GDPv2_659	SSD_069	T. durum	landrace	2.94	1.19	0.97
GDPv2_660	SSD_092	T. durum	landrace	1.32	0.82	1.28
GDPv2_661	SSD_107	T. durum	landrace	2.04	1.17	1.54
GDPv2_662	SSD_109	T. durum	landrace	1.42	1.69	1.57
GDPv2_663	SSD_111	T. durum	landrace	2.50	1.57	0.81
GDPv2_664	SSD_112	T. durum	landrace	2.89	1.55	2.49
GDPv2_666	SSD_115	T. durum	landrace	1.78	2.48	1.48
GDPv2_667	SSD_118	T. durum	landrace	2.37	1.61	1.01

GDPv2_668	SSD_128	T. durum	landrace	3.61	1.01	0.79
GDPv2_669	SSD_137	T. durum	landrace	1.96	1.31	0.99
GDPv2_670	SSD_142	T. durum	landrace	3.18	1.43	2.06
GDPv2_671	SSD_155	T. durum	landrace	1.60	3.43	2.15
GDPv2_672	SSD_157	T. durum	landrace	2.20	0.99	1.16
GDPv2_673	SSD_158	T. durum	landrace	2.37	1.14	1.45
GDPv2_674	SSD_162	T. durum	landrace	2.47	2.63	2.02
GDPv2_675	SSD_173	T. durum	landrace	3.24	3.20	2.83
GDPv2_677	SSD_182	T. durum	landrace	2.52	2.53	1.91
GDPv2_678	SSD_188	T. durum	landrace	2.20	0.85	1.44
GDPv2_679	SSD_191	T. durum	landrace	2.32	1.08	0.56
GDPv2_680	SSD_195	T. durum	landrace	1.12	0.77	1.25
GDPv2_681	SSD_239	T. durum	landrace	2.87	0.79	1.43
GDPv2_683	SSD_244	T. durum	landrace	2.58	0.91	0.99
GDPv2_685	SSD_253	T. durum	landrace	2.07	1.19	1.14
GDPv2_686	SSD_255	T. durum	landrace	1.49	0.61	2.00
GDPv2_687	SSD_256	T. durum	landrace	2.23	1.61	2.04
GDPv2_688	SSD_262	T. durum	landrace	2.11	1.31	0.86
GDPv2_689	SSD_264	T. durum	landrace	2.13	0.94	1.27
GDPv2_692	SSD_278	T. durum	landrace	1.39	1.09	0.88
GDPv2_693	SSD_292	T. durum	landrace	2.92	1.01	1.70
GDPv2_694	SSD_294	T. durum	landrace	2.32	1.52	1.10
GDPv2_695	SSD_302	T. durum	landrace	2.04	0.57	0.72
GDPv2_696	SSD_303	T. durum	landrace	1.71	1.08	0.82
GDPv2_697	SSD_315	T. durum	landrace	2.23	1.09	0.95
GDPv2_698	SSD_322	T. durum	landrace	1.63	1.44	2.40
GDPv2_699	SSD_325	T. durum	landrace	2.53	2.03	2.30
GDPv2_701	SSD_336	T. durum	landrace	1.50	1.02	1.34
GDPv2_702	SSD_348	T. durum	landrace	2.20	1.21	1.41
GDPv2_704	SSD_407	T. durum	landrace	3.31	2.22	0.95
GDPv2_705	SSD_411	T. durum	landrace	1.61	0.85	1.10
GDPv2_706	SSD_412	T. durum	landrace	1.41	1.90	1.70
GDPv2_707	SSD_415	T. durum	landrace	2.28	1.01	0.87
GDPv2_708	SSD_416	T. durum	landrace	1.93	1.87	1.51
GDPv2_709	SSD_431	T. durum	landrace	1.83	1.44	1.48
GDPv2_710	SSD_443	T. durum	landrace	2.38	1.98	2.19
GDPv2_711	SSD_451	T. durum	landrace	2.29	1.20	1.10
GDPv2_714	SSD_470	T. durum	landrace	2.56	0.94	1.04
GDPv2_715	SSD_477	T. durum	landrace	1.82	1.58	1.24
GDPv2_716	SSD_482	T. durum	landrace	2.18	1.12	1.92
GDPv2_717	SSD_483	T. durum	landrace	1.94	1.85	1.69
GDPv2_719	SSD_491	T. durum	landrace	1.70	1.91	1.46
GDPv2_720	SSD_500	T. durum	landrace	0.90	0.38	0.44
GDPv2_721	SSD_531	T. durum	landrace	2.73	1.99	2.60
GDPv2_722	BIANCUCCIA	T. durum	landrace	1.46	1.38	0.78
GDPv2_723	BIVONA	T. turgidum	landrace	2.33	1.42	1.22
GDPv2_724	BUFALA_Rossa_Lunga	T. turgidum	landrace	2.16	1.96	1.27
GDPv2_725	CASTIGLIONE	T. durum	landrace	2.26	2.00	1.67
GDPv2_726	CICIREDDA	T. turgidum	landrace	2.47	2.32	1.45
GDPv2_727	CURCITTA	T. durum	landrace	2.43	1.86	1.23
GDPv2_728	FARRO_LUNGO	T. durum	landrace	2.05	2.29	1.56
GDPv2_729	GIOIA	T. durum	landrace	1.34	0.66	0.89
GDPv2_730	INGLESA	T. durum	landrace	2.61	2.59	2.17
GDPv2_731	LINA	T. durum	landrace	1.82	1.65	2.03
GDPv2_732	PAOLA	T. durum	landrace	2.27	1.45	1.36
GDPv2_734	SAMMARTINARA	T. durum	landrace	2.52	1.68	1.51
GDPv2_736	CANU_ARDESU	T. durum	landrace	3.10	2.43	1.81
GDPv2_737	CANU_BASCIU	T. durum	landrace	2.28	1.02	0.96
GDPv2_745	PI480352	T. durum	landrace	2.70	2.19	0.63
GDPv2_746	PI204020_Vermelho de Barba Preta	T. durum	landrace	4.38	2.37	1.79

GDPv2_747	Aziziah	T. durum	landrace	1.50	1.26	1.35
GDPv2_748	Kyperounda	T. durum	landrace	2.56	0.87	1.10
GDPv2_749	Taganrog	T. durum	landrace	1.30	0.64	1.46
GDPv2_750	Timilia	T. durum	landrace	2.48	0.97	0.88
GDPv2_752	377b	T. durum	landrace	1.41	0.97	0.73
GDPv2_754	Akbasak	T. durum	landrace	2.12	1.46	1.65
GDPv2_755	Azulejo	T. durum	landrace	1.60	0.93	0.76
GDPv2_757	Hordeiforme_27	T. durum	landrace	1.73	1.23	0.97
GDPv2_758	Abyssinicum	T. durum	landrace	1.55	1.25	0.99
GDPv2_760	T-2	T. durum	landrace	1.69	0.94	0.88
GDPv2_761	IQ_118	T. durum	landrace	2.39	1.55	2.52
GDPv2_762	Mamhoudi	T. durum	landrace	2.10	0.97	0.97
GDPv2_763	IWA8609000	T. turanicum	landrace	2.32	1.50	0.95
GDPv2_552	Kilinto	T. durum	cv	1.78	0.94	1.12
GDPv2_554	Robe	T. durum	cv	1.70	1.00	1.21
GDPv2_555	Boohai	T. durum	cv	1.27	1.15	0.85
GDPv2_570	Almaz	T. durum	cv	1.97	2.48	0.72
GDPv2_571	Kustanayskaya_12	T. durum	cv	2.25	1.43	1.88
GDPv2_572	Kustanayskaya_52	T. durum	cv	2.61	1.14	1.15
GDPv2_575	Altaiski_yanvar	T. durum	cv	1.77	0.78	0.75
GDPv2_576	ordeiforme_254	T. durum	cv	3.31	0.93	0.93
GDPv2_577	Karabalykskaya_chemokolosaya_20	T. durum	cv	1.83	0.65	0.89
GDPv2_578	Omski_rubin	T. durum	cv	2.37	1.50	0.75
GDPv2_738	LEUCURUM-3	T. durum	cv	1.59	0.95	1.13
GDPv2_739	MARVARID	T. durum	cv	3.04	1.02	2.24
GDPv2_740	YOQUT-2014	T. durum	cv	1.35	1.23	0.49
GDPv2_741	MINGCHINOR	T. durum	cv	1.77	0.92	1.88
GDPv2_742	ISTIQBOLLI	T. durum	cv	1.30	0.80	1.69
GDPv2_743	MAKUZ-3	T. durum	cv	1.72	1.28	1.52
GDPv2_282	Simeto	T. durum	cv	1.94	0.68	0.95
GDPv2_105	Svevo	T. durum	cv	1.43	0.51	0.50
GDPv2_379	Vendetta	T. durum	cv	1.92	0.65	1.27
GDPv2_347	Aureo	T. durum	cv	3.23	1.63	0.83
GDPv2_069*	meridiano.1	T. durum	cv_Check	1.33	0.49	0.74
GDPv2_069*	meridiano.2	T. durum	cv_Check	1.58	0.63	1.51
GDPv2_069*	meridiano.3	T. durum	cv_Check	1.64	0.72	1.21
GDPv2_069*	meridiano.4	T. durum	cv_Check	1.63	0.50	0.95
GDPv2_069*	meridiano.5	T. durum	cv_Check	1.80	1.36	0.90
GDPv2_069*	meridiano.6	T. durum	cv_Check	NA	1.01	1.17
GDPv2_069*	meridiano.7	T. durum	cv_Check	NA	1.47	0.89
	iran1.1		cv_Check	1.56	0.85	1.15
	iran1.2		cv_Check	2.25	0.62	1.54
	iran1.3		cv_Check	1.45	1.02	1.03
	iran1.4		cv_Check	1.80	1.41	1.42
	iran1.5		cv_Check	1.62	1.09	0.42
	iran1.6		cv_Check	NA	0.53	1.58
	iran1.7		cv_Check	NA	1.45	1.22
	kiperound.1		cv_Check	1.33	1.26	1.01
	kiperound.2		cv_Check	1.48	1.10	0.81
	kiperound.3		cv_Check	1.06	1.59	0.64
	kiperound.4		cv_Check	1.58	1.43	1.37
	kiperound.5		cv_Check	1.33	1.43	0.87
	kiperound.6		cv_Check	1.69	1.84	1.05
	kiperound.7		cv_Check	1.78	1.53	1.03
	kirmizi.1		cv_Check	1.32	1.70	0.76
	kirmizi.2		cv_Check	1.69	1.26	0.57
	kirmizi.3		cv_Check	1.83	1.16	0.57
	kirmizi.4		cv_Check	1.68	NA	1.13
	kirmizi.5		cv_Check	2.21	0.95	1.01
	kirmizi.6		cv_Check	1.69	0.26	0.83

	kirmizi.7		cv_Check	NA	1.15	0.79
	mindum.1		cv_Check	1.55	1.73	0.92
	mindum.2		cv_Check	1.80	1.39	1.12
	mindum.3		cv_Check	1.62	1.44	0.83
	mindum.4		cv_Check	2.36	1.32	1.00
	mindum.5		cv_Check	1.51	2.08	0.70
	mindum.6		cv_Check	NA	1.44	1.09
	mindum.7		cv_Check	NA	NA	0.87

Table S3.6: ANOVA to evaluate differences across years in free asparagine accumulation.

SUMMARY three years						
Groups	Count	Sum	Average	Variance		
fAsn_2020	201	431.3070011	2.145805976	0.425657652		
fAsn_2021	201	284.7117453	1.416476345	0.387577744		
fAsn_2022	201	264.0042789	1.313454124	0.315272399		
VARIANCE ANALYSIS						
Source of variation	SQ	df	MS	F	P-value	F crit
Tra gruppi	82.76810789	2	41.38405395	110.0144476	1.97983E-41	3.010739538
In gruppi	225.7015592	600	0.376169265			
Totale	308.4696671	602				

SUMMARY 2020vs2021						
Groups	Count	Sum	Average	Variance		
fAsn_2020	201	431.3070011	2.145805976	0.425657652		
fAsn_2021	201	284.7117453	1.416476345	0.387577744		
VARIANCE ANALYSIS						
Source of variation	SQ	df	MS	F	P-value	F crit
Tra gruppi	53.45813191	1	53.45813191	131.4702536	1.64795E-26	3.864811341
In gruppi	162.6470794	400	0.406617698			
Totale	216.1052113	401				

SUMMARY 2020vs2022						
Groups	Count	Sum	Average	Variance		
fAsn_2020	201	431.3070011	2.145805976	0.425657652		
fAsn_2022	201	264.0042789	1.313454124	0.315272399		
VARIANCE ANALYSIS						
Source of variation	SQ	df	MS	F	P-value	F crit
Tra gruppi	69.62736534	1	69.62736534	187.9458531	2.45784E-35	3.864811341
In gruppi	148.1860103	400	0.370465026			
Totale	217.8133757	401				

SUMMARY 2021vs2022						
Groups	Count	Sum	Average	Variance		
fAsn_2021	201	284.7117453	1.416476345	0.387577744		
fAsn_2022	201	264.0042789	1.313454124	0.315272399		
VARIANCE ANALYSIS						
Source of variation	SQ	df	MS	F	P-value	F crit
Tra gruppi	1.066664591	1	1.066664591	3.035254673	0.082242369	3.864811341
In gruppi	140.5700287	400	0.351425072			
Totale	141.6366933	401				

Table S3.7: Free asparagine measurements by NMR.

GDP Accession	fAsn (mmol/Kg) year 2020
GDPv2_069 (meridiano.1)	1.98
GDPv2_091	2.19
GDPv2_105	1.95
GDPv2_347	3.62
GDPv2_550	3.06
GDPv2_551	3.22
GDPv2_554	2.23
GDPv2_555	1.68
GDPv2_556	1.76
GDPv2_557	2.06
GDPv2_561	2.33
GDPv2_563	1.94
GDPv2_565	3.32
GDPv2_572	3.06
GDPv2_576	3.41
GDPv2_580	3.34
GDPv2_581	4.17
GDPv2_583	3.15
GDPv2_586	2.28
GDPv2_587	2.52
GDPv2_588	3.64
GDPv2_589	1.70
GDPv2_592	3.75
GDPv2_594	3.69
GDPv2_595	2.71
GDPv2_597	3.04
GDPv2_598	1.77
GDPv2_600	1.50
GDPv2_601	2.72
GDPv2_602	1.92
GDPv2_610	3.69
GDPv2_614	1.88
GDPv2_615	1.75
GDPv2_618	1.79
GDPv2_620	2.60
GDPv2_622	3.15
GDPv2_623	4.39
GDPv2_625	3.04
GDPv2_626	1.46
GDPv2_628	1.64
GDPv2_629	1.34
GDPv2_632	3.91

GDPv2_633	4.23
GDPv2_634	2.94
GDPv2_635	1.63
GDPv2_636	1.93
GDPv2_637	1.66
GDPv2_640	1.87
GDPv2_641	3.48
GDPv2_644	1.29
GDPv2_645	1.49
GDPv2_646	3.03
GDPv2_648	1.38
GDPv2_653	1.70
GDPv2_659	3.70
GDPv2_660	1.27
GDPv2_664	2.71
GDPv2_665	1.76
GDPv2_670	4.10
GDPv2_675	3.54
GDPv2_677	3.19
GDPv2_681	3.12
GDPv2_684	7.00
GDPv2_686	1.83
GDPv2_687	2.31
GDPv2_692	1.49
GDPv2_693	3.31
GDPv2_696	1.68
GDPv2_699	2.78
GDPv2_701	1.66
GDPv2_704	3.59
GDPv2_705	1.90
GDPv2_706	1.67
GDPv2_714	2.84
GDPv2_719	2.11
GDPv2_720	1.40
GDPv2_721	3.52
GDPv2_722	2.01
GDPv2_726	2.17
GDPv2_729	1.49
GDPv2_730	2.78
GDPv2_733	2.66
GDPv2_734	2.55
GDPv2_736	4.07
GDPv2_738	2.23
GDPv2_740	1.49
GDPv2_742	1.51
GDPv2_743	1.91
GDPv2_746	5.13
GDPv2_747	1.91
GDPv2_748	2.97
GDPv2_750	2.90
GDPv2_752	1.44
GDPv2_755	1.85
GDPv2_758	1.76
GDPv2_760	1.88
kirmizi.1	1.91
kiperound.1	2.25

Table S3.8: High free asparagine content clade. Genotypes in red shown free asparagine median values higher than 1.39 mmol/kg (entire collection median value across 3 year).

n°	FINAL accession code	Country of collection / development	Macroarea	fAsn median value across 3 years (mmol/kg)*
1	GDPv2_674	SAU	West Asia	2.47
2	GDPv2_549	EGY	North Africa	1.93
3	GDPv2_550	EGY	North Africa	2.54
4	GDPv2_548	EGY	North Africa	1.48
5	GDPv2_698	TUR	West Asia	1.63
6	GDPv2_1000	IND	South Asia	2.30
7	GDPv2_670	PRT	Iberica peninsula	2.06
8	GDPv2_675	EGY	North Africa	3.20
9	GDPv2_609	YEM	West Asia	1.69
10	GDPv2_608	YEM	West Asia	1.35
11	GDPv2_551	EGY	North Africa	2.31
12	GDPv2_717	GRC	Balkans	1.85
13	GDPv2_621	YUG	Balkans	1.27
14	GDPv2_560	TUR	West Asia	1.86
15	GDPv2_746	PRT	Iberica peninsula	2.37
16	GDPv2_677	IND	South Asia	2.52
17	GDPv2_595	ETH	Horn of Africa	3.00
18	GDPv2_594	ETH	Horn of Africa	2.80
19	GDPv2_596	ETH	Horn of Africa	2.66
20	GDPv2_625	ETH	Horn of Africa	2.52
21	GDPv2_582	ESP	Iberica peninsula	1.63
22	GDPv2_745	ETH	Horn of Africa	2.19
23	GDPv2_623	ETH	Horn of Africa	1.84
24	GDPv2_622	ETH	Horn of Africa	2.39
25	GDPv2_617	ETH	Horn of Africa	0.76
26	GDPv2_673	ETH	Horn of Africa	1.45
27	GDPv2_672	TUN	North Africa	1.16
28	GDPv2_607	YEM	West Asia	2.00
29	GDPv2_694	TUN	North Africa	1.52
30	GDPv2_601	ETH	Horn of Africa	2.64
31	GDPv2_592	ETH	Horn of Africa	2.10
32	GDPv2_616	OMN	West Asia	1.68
33	GDPv2_591	OMN	West Asia	1.19
34	GDPv2_619	SAU	West Asia	0.99
35	GDPv2_727	ITA	Italy	1.86
36	GDPv2_726	ITA	Italy	2.32
37	GDPv2_587	AFG	Central Asia	2.79
38	GDPv2_586	GRC	Balkans	2.63
39	GDPv2_732	ITA	Italy	1.45

Table S3.9: GAPIT results.

SNP	Chr	Pos	P.value	MAF	nobs	H&B.P.Value	Effect	log10(p)	alg.
BobWhite_c5242_400	2	635342239	0.000998877	0.124378109	196	0.130992453	NA	3.000488057	SUPER
BobWhite_c7333_76	2	309807820	0.000352156	0.467661692	201	0.109847729	- 0.108569578	3.453264597	GLM
BobWhite_c7333_76	2	309807820	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
BobWhite_c7888_145	4	729015353	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
BobWhite_rep_c65414_125	4	728962900	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
BobWhite_rep_c65414_318	4	728963093	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
BobWhite_rep_c67106_132	4	728963266	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
BS00003494_51	14	544859412	0.000335844	0.303482587	201	0.491955937	0.133088614	3.473861838	BLINK
BS00003494_51	14	544859412	0.000343707	0.303482587	201	0.906558536	0.137807745	3.463811775	CMLM
BS00003494_51	14	544859412	0.000335844	0.303482587	201	0.491955937	0.133088614	3.473861838	FarmCPU
BS00003494_51	14	544859412	5.76E-05	0.303482587	201	0.098074433	0.133088614	4.239877101	GLM
BS00003494_51	14	544859412	0.000343707	0.303482587	201	0.906558536	0.137807745	3.463811775	MLM
BS00003494_51	14	544859412	0.000221051	0.303482587	201	0.898827307	0.13780774	3.655506934	MLMM
BS00003494_51	14	544859412	1.33E-05	0.303482587	196	0.074075607	NA	4.877167575	SUPER
BS00012230_51	4	730169348	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
BS00022249_51	2	624211720	0.000925947	0.134328358	201	0.155341851	- 0.150932001	3.033414004	GLM
BS00022249_51	2	624211720	0.000429868	0.134328358	196	0.092721797	NA	3.36666511	SUPER
BS00029224_51	3	556826479	0.000265666	0.099502488	201	0.109847729	-0.1909205	3.575663335	GLM
BS00029224_51	3	556826479	0.000256204	0.099502488	196	0.074075607	NA	3.59141442	SUPER
BS00034450_51	2	623410947	0.000615967	0.139303483	201	0.123093842	- 0.154770023	3.210442258	GLM
BS00034450_51	2	623410947	0.000248308	0.139303483	196	0.074075607	NA	3.605009454	SUPER
BS00062942_51	2	308056159	0.000352156	0.467661692	201	0.109847729	0.108569578	3.453264597	GLM
BS00062942_51	2	308056159	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
BS00064436_51	4	729812377	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
BS00086227_51	4	728957468	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
BS00086228_51	4	728957459	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
BS00092630_51	14	543768462	0.00060317	0.308457711	201	0.491955937	- 0.126292597	3.219560041	BLINK
BS00092630_51	14	543768462	0.00060317	0.308457711	201	0.491955937	- 0.126292597	3.219560041	FarmCPU
BS00092630_51	14	543768462	0.000110929	0.308457711	201	0.098074433	- 0.126292597	3.954956826	GLM
BS00092630_51	14	543768462	5.14E-05	0.308457711	196	0.074075607	NA	4.288791866	SUPER
BS00108573_51	14	545158872	0.000341745	0.298507463	201	0.491955937	- 0.133954955	3.466298397	BLINK
BS00108573_51	14	545158872	0.000341526	0.298507463	201	0.906558536	- 0.138564387	3.466576413	CMLM
BS00108573_51	14	545158872	0.000341745	0.298507463	201	0.491955937	- 0.133954955	3.466298397	FarmCPU
BS00108573_51	14	545158872	5.87E-05	0.298507463	201	0.098074433	- 0.133954955	4.231459233	GLM
BS00108573_51	14	545158872	0.000341526	0.298507463	201	0.906558536	- 0.138564387	3.466576413	MLM
BS00108573_51	14	545158872	0.00021946	0.298507463	201	0.898827307	- 0.138564383	3.658645263	MLMM
BS00108573_51	14	545158872	1.46E-05	0.298507463	196	0.074075607	NA	4.835843633	SUPER
CAP7_rep_c12580_266	2	307095419	0.000509265	0.472636816	201	0.109847729	0.105556993	3.293055868	GLM
CAP7_rep_c12580_266	2	307095419	0.000342832	0.472636816	196	0.081732359	NA	3.464918608	SUPER
Excalibur_c11031_129	4	730168241	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
Excalibur_c13046_166	4	728957368	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
Excalibur_c17745_108	4	730170198	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
Excalibur_c20228_135	2	297859361	0.000784679	0.348258706	201	0.491955937	- 0.117456961	3.105307936	BLINK
Excalibur_c20228_135	2	297859361	0.000784679	0.348258706	201	0.491955937	- 0.117456961	3.105307936	FarmCPU

Excalibur_c20228_135	2	297859361	0.000149393	0.348258706	201	0.098074433	- 0.117456961	3.825670696	GLM
Excalibur_c20228_135	2	297859361	0.000145118	0.348258706	196	0.074075607	NA	3.838278738	SUPER
Excalibur_c27675_1815	2	317189649	0.000731567	0.348258706	201	0.138073103	- 0.104438567	3.135745917	GLM
Excalibur_c27675_912	2	317190770	0.000504065	0.393034826	201	0.109847729	- 0.104594468	3.297513501	GLM
Excalibur_c27675_912	2	317190770	0.000423023	0.393034826	196	0.09271714	NA	3.373635784	SUPER
Excalibur_c4082_370	2	308051120	0.000352156	0.467661692	201	0.109847729	- 0.108569578	3.453264597	GLM
Excalibur_c4082_370	2	308051120	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
Excalibur_c52990_401	4	730211959	0.000534166	0.104477612	201	0.11341856	- 0.169723104	3.272323396	GLM
Excalibur_c52990_401	4	730211959	0.000248197	0.104477612	196	0.074075607	NA	3.605202757	SUPER
GENE-0769_164	4	728958360	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
GENE-1232_258	4	730171192	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
IAAV1142	1	509420913	0.000350219	0.313432836	201	0.109847729	0.12453515	3.45565999	GLM
IAAV1142	1	509420913	0.000363925	0.313432836	196	0.085265176	NA	3.438987814	SUPER
IAAV5269	2	295660272	0.000245283	0.39800995	201	0.109847729	- 0.115258943	3.61033175	GLM
IAAV5269	2	295660272	0.000185934	0.39800995	196	0.074075607	NA	3.7306423	SUPER
IAAV8661	2	295362696	0.000245283	0.39800995	201	0.109847729	0.115258943	3.61033175	GLM
IAAV8661	2	295362696	0.000185934	0.39800995	196	0.074075607	NA	3.7306423	SUPER
IACX1339	2	307087306	0.000330248	0.388059701	201	0.109847729	- 0.108379336	3.48116041	GLM
IACX1339	2	307087306	0.000447432	0.388059701	196	0.093277966	NA	3.349272761	SUPER
IACX6070	10	597749316	0.000773366	0.243781095	196	0.114774022	NA	3.11161505	SUPER
JD_c2834_381	2	301935474	0.000497089	0.333333333	201	0.109847729	- 0.109973548	3.303565936	GLM
JD_c2834_381	2	301935474	0.000781178	0.333333333	196	0.114774022	NA	3.107249841	SUPER
Ku_c1485_980	5	506385315	0.000185926	0.179104478	196	0.074075607	NA	3.73065948	SUPER
Ku_c62848_956	2	307265205	0.000509265	0.472636816	201	0.109847729	0.105556993	3.293055868	GLM
Ku_c62848_956	2	307265205	0.000342832	0.472636816	196	0.081732359	NA	3.464918608	SUPER
Kukri_c10308_342	2	290975897	0.000207646	0.333333333	201	0.109847729	0.116073318	3.682675775	GLM
Kukri_c10308_342	2	290975897	0.000209462	0.333333333	196	0.074075607	NA	3.678894402	SUPER
Kukri_c11564_481	4	730228355	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
Kukri_c2535_98	2	307086718	0.000509265	0.472636816	201	0.109847729	- 0.105556993	3.293055868	GLM
Kukri_c2535_98	2	307086718	0.000342832	0.472636816	196	0.081732359	NA	3.464918608	SUPER
Kukri_c32052_171	1	8967248	0.000813686	0.213930348	201	0.141237117	- 0.121343056	3.089542941	GLM
Kukri_c32885_145	1	332149558	0.000777682	0.189054726	201	0.141237117	0.161683569	3.109198189	GLM
Kukri_c44509_515	1	509613302	0.0008318	0.328358209	201	0.491955937	0.130966623	3.079981127	BLINK
Kukri_c44509_515	1	509613302	0.0008318	0.328358209	201	0.491955937	0.130966623	3.079981127	FarmCPU
Kukri_c44509_515	1	509613302	0.000159624	0.328358209	201	0.098596545	0.130966623	3.796903119	GLM
Kukri_c44509_515	1	509613302	0.000194271	0.328358209	196	0.074075607	NA	3.711591728	SUPER
Kukri_c45663_496	2	315939055	0.000692886	0.402985075	201	0.13450903	0.10151364	3.159338019	GLM
Kukri_c45663_496	2	315939055	0.000592889	0.402985075	196	0.106878225	NA	3.227026433	SUPER
Kukri_c62848_106	2	307267970	0.000944828	0.492537313	196	0.127121462	NA	3.024647247	SUPER
Kukri_c62848_1581	2	307263578	0.000509265	0.472636816	201	0.109847729	- 0.105556993	3.293055868	GLM
Kukri_c62848_1581	2	307263578	0.000342832	0.472636816	196	0.081732359	NA	3.464918608	SUPER
Kukri_c62848_1632	2	307263564	0.000509265	0.472636816	201	0.109847729	- 0.105556993	3.293055868	GLM
Kukri_c62848_1632	2	307263564	0.000342832	0.472636816	196	0.081732359	NA	3.464918608	SUPER
Kukri_c7428_604	2	309236634	0.000381192	0.462686567	201	0.109847729	0.107681151	3.418855884	GLM
Kukri_c7428_604	2	309236634	0.000254865	0.462686567	196	0.074075607	NA	3.593690314	SUPER
Kukri_rep_c106406_265	2	317456540	0.000504065	0.393034826	201	0.109847729	- 0.104594468	3.297513501	GLM
Kukri_rep_c106406_265	2	317456540	0.000423023	0.393034826	196	0.09271714	NA	3.373635784	SUPER
Kukri_rep_c111517_289	2	317189792	0.000504065	0.393034826	201	0.109847729	0.104594468	3.297513501	GLM

Kukri_rep_c111517_289	2	317189792	0.000423023	0.393034826	196	0.09271714	NA	3.373635784	SUPER
Kukri_rep_c68583_931	2	307265067	0.000509265	0.472636816	201	0.109847729	0.105556993	3.293055868	GLM
Kukri_rep_c68583_931	2	307265067	0.000342832	0.472636816	196	0.081732359	NA	3.464918608	SUPER
Ra_c106383_270	4	446141376	0.000625082	0.437810945	196	0.106878225	NA	3.20406305	SUPER
Ra_c1195_200	2	295095363	0.000245283	0.39800995	201	0.109847729	0.115258943	3.61033175	GLM
Ra_c1195_200	2	295095363	0.000185934	0.39800995	196	0.074075607	NA	3.7306423	SUPER
RAC875_c14006_93	12	641110379	0.000809621	0.094527363	201	0.141237117	0.169086544	3.091718264	GLM
RAC875_c14926_589	1	25527210	0.000372574	0.094527363	201	0.109847729	- 0.250829662	3.428787709	GLM
RAC875_c14926_589	1	25527210	0.000449357	0.094527363	196	0.093277966	NA	3.347408666	SUPER
RAC875_c16677_56	2	308051848	0.000352156	0.467661692	201	0.109847729	0.108569578	3.453264597	GLM
RAC875_c16677_56	2	308051848	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
RAC875_c20420_87	2	309231907	0.000352156	0.467661692	201	0.109847729	- 0.108569578	3.453264597	GLM
RAC875_c20420_87	2	309231907	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
RAC875_c21131_3615	2	294801600	0.000523008	0.338308458	201	0.491955937	0.121833784	3.281491782	BLINK
RAC875_c21131_3615	2	294801600	0.000523008	0.338308458	201	0.491955937	0.121833784	3.281491782	FarmCPU
RAC875_c21131_3615	2	294801600	9.45E-05	0.338308458	201	0.098074433	0.121833784	4.024705569	GLM
RAC875_c21131_3615	2	294801600	7.67E-05	0.338308458	196	0.074075607	NA	4.114962973	SUPER
RAC875_c50365_521	2	294633354	0.000794703	0.343283582	201	0.491955937	- 0.117595043	3.099795218	BLINK
RAC875_c50365_521	2	294633354	0.000794703	0.343283582	201	0.491955937	- 0.117595043	3.099795218	FarmCPU
RAC875_c50365_521	2	294633354	0.000151561	0.343283582	201	0.098074433	- 0.117595043	3.819412397	GLM
RAC875_c50365_521	2	294633354	0.000128597	0.343283582	196	0.074075607	NA	3.890770324	SUPER
RAC875_rep_c108665_52	4	728963721	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
RAC875_rep_c110216_250	2	294803710	0.000523008	0.338308458	201	0.491955937	0.121833784	3.281491782	BLINK
RAC875_rep_c110216_250	2	294803710	0.000523008	0.338308458	201	0.491955937	0.121833784	3.281491782	FarmCPU
RAC875_rep_c110216_250	2	294803710	9.45E-05	0.338308458	201	0.098074433	0.121833784	4.024705569	GLM
RAC875_rep_c110216_250	2	294803710	7.67E-05	0.338308458	196	0.074075607	NA	4.114962973	SUPER
RAC875_rep_c112205_166	9	532077979	0.000855671	0.109452736	196	0.122396959	NA	3.067693266	SUPER
RAC875_rep_c117462_124	2	308052122	0.000352156	0.467661692	201	0.109847729	0.108569578	3.453264597	GLM
RAC875_rep_c117462_124	2	308052122	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
RAC875_rep_c75762_175	2	307265166	0.000509265	0.472636816	201	0.109847729	- 0.105556993	3.293055868	GLM
RAC875_rep_c75762_175	2	307265166	0.000342832	0.472636816	196	0.081732359	NA	3.464918608	SUPER
RFL_Contig1354_484	2	307905179	0.000352156	0.467661692	201	0.109847729	- 0.108569578	3.453264597	GLM
RFL_Contig1354_484	2	307905179	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
RFL_Contig1354_663	2	307905358	0.000352156	0.467661692	201	0.109847729	0.108569578	3.453264597	GLM
RFL_Contig1354_663	2	307905358	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
RFL_Contig2784_641	2	309808136	0.000352156	0.467661692	201	0.109847729	- 0.108569578	3.453264597	GLM
RFL_Contig2784_641	2	309808136	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
RFL_Contig3273_298	4	730225739	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
TA001523-1009	9	618268210	0.000337429	0.069651741	201	0.109847729	0.246145672	3.471818032	GLM
TA001523-1009	9	618268210	0.000818398	0.069651741	201	0.898827307	0.254151019	3.087035246	MLMM
TA001523-1009	9	618268210	0.000453039	0.069651741	196	0.093277966	NA	3.343864497	SUPER
TA004184-0525	2	293858246	0.000784679	0.348258706	201	0.491955937	0.117456961	3.105307936	BLINK
TA004184-0525	2	293858246	0.000784679	0.348258706	201	0.491955937	0.117456961	3.105307936	FarmCPU
TA004184-0525	2	293858246	0.000149393	0.348258706	201	0.098074433	0.117456961	3.825670696	GLM
TA004184-0525	2	293858246	0.000145118	0.348258706	196	0.074075607	NA	3.838278738	SUPER
Tdurum_contig10268_1000	10	555635994	0.0007057	0.457711443	201	0.135067048	0.114362908	3.151379742	GLM
Tdurum_contig10572_580	5	506414435	0.000185926	0.179104478	196	0.074075607	NA	3.73065948	SUPER
Tdurum_contig11660_328	2	305596669	0.000216051	0.383084577	201	0.109847729	- 0.112125878	3.665442806	GLM
Tdurum_contig11660_328	2	305596669	0.000284489	0.383084577	196	0.080540059	NA	3.545934317	SUPER
Tdurum_contig13391_680	2	297859203	0.000784679	0.348258706	201	0.491955937	- 0.117456961	3.105307936	BLINK

Tdurum_contig13391_680	2	297859203	0.000784679	0.348258706	201	0.491955937	- 0.117456961	3.105307936	FarmCPU
Tdurum_contig13391_680	2	297859203	0.000149393	0.348258706	201	0.098074433	- 0.117456961	3.825670696	GLM
Tdurum_contig13391_680	2	297859203	0.000145118	0.348258706	196	0.074075607	NA	3.838278738	SUPER
Tdurum_contig288_253	2	294389869	0.000794703	0.343283582	201	0.491955937	- 0.117595043	3.099795218	BLINK
Tdurum_contig288_253	2	294389869	0.000794703	0.343283582	201	0.491955937	- 0.117595043	3.099795218	FarmCPU
Tdurum_contig288_253	2	294389869	0.000151561	0.343283582	201	0.098074433	- 0.117595043	3.819412397	GLM
Tdurum_contig288_253	2	294389869	0.000128597	0.343283582	196	0.074075607	NA	3.890770324	SUPER
Tdurum_contig29769_202	2	317190630	0.000504065	0.393034826	201	0.109847729	0.104594468	3.297513501	GLM
Tdurum_contig29769_202	2	317190630	0.000423023	0.393034826	196	0.09271714	NA	3.373635784	SUPER
Tdurum_contig31201_201	2	307905458	0.000352156	0.467661692	201	0.109847729	0.108569578	3.453264597	GLM
Tdurum_contig31201_201	2	307905458	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
Tdurum_contig31201_380	2	307905279	0.000352156	0.467661692	201	0.109847729	- 0.108569578	3.453264597	GLM
Tdurum_contig31201_380	2	307905279	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
Tdurum_contig42289_1857	2	298638602	0.000784679	0.348258706	201	0.491955937	- 0.117456961	3.105307936	BLINK
Tdurum_contig42289_1857	2	298638602	0.000784679	0.348258706	201	0.491955937	- 0.117456961	3.105307936	FarmCPU
Tdurum_contig42289_1857	2	298638602	0.000149393	0.348258706	201	0.098074433	- 0.117456961	3.825670696	GLM
Tdurum_contig42289_1857	2	298638602	0.000145118	0.348258706	196	0.074075607	NA	3.838278738	SUPER
Tdurum_contig42289_936	2	298643140	0.000784679	0.348258706	201	0.491955937	0.117456961	3.105307936	BLINK
Tdurum_contig42289_936	2	298643140	0.000784679	0.348258706	201	0.491955937	0.117456961	3.105307936	FarmCPU
Tdurum_contig42289_936	2	298643140	0.000149393	0.348258706	201	0.098074433	0.117456961	3.825670696	GLM
Tdurum_contig42289_936	2	298643140	0.000145118	0.348258706	196	0.074075607	NA	3.838278738	SUPER
Tdurum_contig43346_108	2	302038083	0.000497089	0.333333333	201	0.109847729	0.109973548	3.303565936	GLM
Tdurum_contig43346_108	2	302038083	0.000781178	0.333333333	196	0.114774022	NA	3.107249841	SUPER
Tdurum_contig47605_1362	14	544281422	0.000497572	0.268656716	201	0.109847729	0.118875039	3.303144496	GLM
Tdurum_contig47605_1362	14	544281422	9.98E-05	0.268656716	196	0.074075607	NA	4.001068051	SUPER
Tdurum_contig48396_341	2	303478652	0.000627415	0.343283582	201	0.123564322	- 0.106817217	3.202445318	GLM
Tdurum_contig48396_341	2	303478652	0.000843609	0.343283582	196	0.121955295	NA	3.073858985	SUPER
Tdurum_contig50581_125	10	545137743	0.000801636	0.273631841	201	0.141237117	- 0.128450911	3.096022767	GLM
Tdurum_contig50581_379	10	545137489	0.000801636	0.273631841	201	0.141237117	0.128450911	3.096022767	GLM
Tdurum_contig52688_476	10	597749309	0.000773366	0.243781095	196	0.114774022	NA	3.11161505	SUPER
Tdurum_contig60809_255	2	301135352	0.000725058	0.353233831	201	0.491955937	- 0.117739843	3.13962744	BLINK
Tdurum_contig60809_255	2	301135352	0.000725058	0.353233831	201	0.491955937	- 0.117739843	3.13962744	FarmCPU
Tdurum_contig60809_255	2	301135352	0.000136587	0.353233831	201	0.098074433	- 0.117739843	3.864589973	GLM
Tdurum_contig60809_255	2	301135352	0.00013642	0.353233831	196	0.074075607	NA	3.865122258	SUPER
Tdurum_contig60809_268	2	301135339	0.000725058	0.353233831	201	0.491955937	0.117739843	3.13962744	BLINK
Tdurum_contig60809_268	2	301135339	0.000725058	0.353233831	201	0.491955937	0.117739843	3.13962744	FarmCPU
Tdurum_contig60809_268	2	301135339	0.000136587	0.353233831	201	0.098074433	0.117739843	3.864589973	GLM
Tdurum_contig60809_268	2	301135339	0.00013642	0.353233831	196	0.074075607	NA	3.865122258	SUPER
Tdurum_contig63991_186	2	293857766	0.000784679	0.348258706	201	0.491955937	0.117456961	3.105307936	BLINK
Tdurum_contig63991_186	2	293857766	0.000784679	0.348258706	201	0.491955937	0.117456961	3.105307936	FarmCPU
Tdurum_contig63991_186	2	293857766	0.000149393	0.348258706	201	0.098074433	0.117456961	3.825670696	GLM
Tdurum_contig63991_186	2	293857766	0.000145118	0.348258706	196	0.074075607	NA	3.838278738	SUPER
Tdurum_contig63991_280	2	293857860	0.000784679	0.348258706	201	0.491955937	0.117456961	3.105307936	BLINK
Tdurum_contig63991_280	2	293857860	0.000784679	0.348258706	201	0.491955937	0.117456961	3.105307936	FarmCPU
Tdurum_contig63991_280	2	293857860	0.000149393	0.348258706	201	0.098074433	0.117456961	3.825670696	GLM
Tdurum_contig63991_280	2	293857860	0.000145118	0.348258706	196	0.074075607	NA	3.838278738	SUPER

Tdurum_contig63991_404	2	293857984	0.000784679	0.348258706	201	0.491955937	- 0.117456961	3.105307936	BLINK
Tdurum_contig63991_404	2	293857984	0.000784679	0.348258706	201	0.491955937	- 0.117456961	3.105307936	FarmCPU
Tdurum_contig63991_404	2	293857984	0.000149393	0.348258706	201	0.098074433	- 0.117456961	3.825670696	GLM
Tdurum_contig63991_404	2	293857984	0.000145118	0.348258706	196	0.074075607	NA	3.838278738	SUPER
Tdurum_contig65853_242	2	623410585	0.000615967	0.139303483	201	0.123093842	- 0.154770023	3.210442258	GLM
Tdurum_contig65853_242	2	623410585	0.000248308	0.139303483	196	0.074075607	NA	3.605009454	SUPER
Tdurum_contig65853_534	2	623410877	0.000615967	0.139303483	201	0.123093842	- 0.154770023	3.210442258	GLM
Tdurum_contig65853_534	2	623410877	0.000248308	0.139303483	196	0.074075607	NA	3.605009454	SUPER
Tdurum_contig7497_570	2	302734740	0.000497089	0.333333333	201	0.109847729	0.109973548	3.303565936	GLM
Tdurum_contig7497_570	2	302734740	0.000781178	0.333333333	196	0.114774022	NA	3.107249841	SUPER
Tdurum_contig81414_256	12	553462768	0.000584184	0.248756219	201	0.122130398	- 0.134598534	3.23345037	GLM
Tdurum_contig81414_256	12	553462768	0.000912133	0.248756219	196	0.123949735	NA	3.039941895	SUPER
tplb0023b14_704	2	438289598	0.000900252	0.109452736	196	0.123570955	NA	3.045635901	SUPER
tplb0024i16_1177	2	299978233	0.000784679	0.348258706	201	0.491955937	0.117456961	3.105307936	BLINK
tplb0024i16_1177	2	299978233	0.000784679	0.348258706	201	0.491955937	0.117456961	3.105307936	FarmCPU
tplb0024i16_1177	2	299978233	0.000149393	0.348258706	201	0.098074433	0.117456961	3.825670696	GLM
tplb0024i16_1177	2	299978233	0.000145118	0.348258706	196	0.074075607	NA	3.838278738	SUPER
tplb0024i16_800	2	299977633	0.000784679	0.348258706	201	0.491955937	- 0.117456961	3.105307936	BLINK
tplb0024i16_800	2	299977633	0.000784679	0.348258706	201	0.491955937	- 0.117456961	3.105307936	FarmCPU
tplb0024i16_800	2	299977633	0.000149393	0.348258706	201	0.098074433	- 0.117456961	3.825670696	GLM
tplb0024i16_800	2	299977633	0.000145118	0.348258706	196	0.074075607	NA	3.838278738	SUPER
tplb0038n17_267	4	730213349	0.000676395	0.084577114	196	0.106878225	NA	3.169799817	SUPER
wsnp_BE497109B_Ta_2_5	2	294390357	0.000794703	0.343283582	201	0.491955937	0.117595043	3.099795218	BLINK
wsnp_BE497109B_Ta_2_5	2	294390357	0.000794703	0.343283582	201	0.491955937	0.117595043	3.099795218	FarmCPU
wsnp_BE497109B_Ta_2_5	2	294390357	0.000151561	0.343283582	201	0.098074433	0.117595043	3.819412397	GLM
wsnp_BE497109B_Ta_2_5	2	294390357	0.000128597	0.343283582	196	0.074075607	NA	3.890770324	SUPER
wsnp_BE517711B_Ta_2_1	10	487492354	0.000507984	0.213930348	201	0.109847729	0.159914979	3.294149899	GLM
wsnp_BE517711B_Ta_2_1	10	487492354	0.000762312	0.213930348	196	0.114774022	NA	3.117867476	SUPER
wsnp_CAP11_c1761_958064	13	585142952	0.000838058	0.243781095	201	0.898827307	- 0.135660874	3.076726129	MLMM
wsnp_CAP8_c954_618139	7	563579589	0.000598165	0.28358209	201	0.491955937	0.133532163	3.223178938	BLINK
wsnp_CAP8_c954_618139	7	563579589	0.000598165	0.28358209	201	0.491955937	0.133532163	3.223178938	FarmCPU
wsnp_CAP8_c954_618139	7	563579589	0.000109891	0.28358209	201	0.098074433	0.133532163	3.959038914	GLM
wsnp_CAP8_c954_618139	7	563579589	0.000141192	0.28358209	196	0.074075607	NA	3.850190821	SUPER
wsnp_Ex_c14_27570	2	305961692	0.000352156	0.467661692	201	0.109847729	- 0.108569578	3.453264597	GLM
wsnp_Ex_c14_27570	2	305961692	0.000225623	0.467661692	196	0.074075607	NA	3.64661653	SUPER
wsnp_Ex_c17452_26163465	2	438545362	0.000900252	0.109452736	196	0.123570955	NA	3.045635901	SUPER
wsnp_Ex_c28481_37607810	10	487493154	0.000831479	0.218905473	201	0.141237117	0.154016578	3.080148667	GLM
wsnp_Ex_c28481_37608092	10	487493404	0.000831479	0.218905473	201	0.141237117	0.154016578	3.080148667	GLM
wsnp_Ex_rep_c67036_65492436	2	438543560	0.000900252	0.109452736	196	0.123570955	NA	3.045635901	SUPER
wsnp_Ex_rep_c69266_68192766	2	299977851	0.000369359	0.402985075	201	0.109847729	- 0.1111695851	3.432551211	GLM
wsnp_Ex_rep_c69266_68192766	2	299977851	0.000293983	0.402985075	196	0.081529301	NA	3.531677699	SUPER
wsnp_JD_c4485_5618761	2	441404447	0.000805857	0.104477612	201	0.141237117	0.193779434	3.093741869	GLM
wsnp_JD_c4485_5618761	2	441404447	0.000505813	0.104477612	196	0.102589439	NA	3.29601004	SUPER
wsnp_Ku_c19037_28455905	14	441175802	0.000785487	0.139303483	196	0.114774022	NA	3.104860947	SUPER
wsnp_Ku_c62848_63784645	2	307267070	0.000509265	0.472636816	201	0.109847729	0.105556993	3.293055868	GLM
wsnp_Ku_c62848_63784645	2	307267070	0.000342832	0.472636816	196	0.081732359	NA	3.464918608	SUPER
wsnp_Ra_c8484_14372772	2	438545016	0.000900252	0.109452736	196	0.123570955	NA	3.045635901	SUPER

Table S3.10.1: TASSEL51 (GLM).

Trcl	Marker	Cv	Pos	marker.F	p	pem.p	marker.Bq	add.F	add.p	dom.F	dom.p	marker.dl	marker.MS	error.dl	error.MS	model.dl	model.MS	microCv	addEffect	domEffect		
IAIN	wrnp_CAPR_c954_618139	7	56357989	19.14437	1.97E-05	0.128	0.06479	19.14437	1.97E-05	NaN	NaN	1	2.8343	195	0.14807	5	2.84213	57	0.14275	NaN	4.70649071	GLM
IAIN	85000264.51	14	54859412	17.83268	3.69E-05	0.216	0.06148	17.83268	3.69E-05	NaN	NaN	1	2.8767	195	0.14997	5	2.86307	61	0.13142	NaN	4.43209788	GLM
IAIN	850010675.51	14	54513872	17.43771	1.47E-05	0.254	0.06041	17.43771	1.47E-05	NaN	NaN	1	2.60267	195	0.14924	5	2.79754	40	0.13133	NaN	4.34932267	GLM
IAIN	kukl_c4439_515	1	33613302	16.9534	4.73E-05	0.356	0.05772	16.9534	4.73E-05	NaN	NaN	1	2.48681	195	0.14985	5	2.77527	66	0.13311	NaN	4.17223767	GLM
IAIN	RACB75_c21131_3415	2	29480160	16.4329	7.26E-05	0.379	0.0672	16.4329	7.26E-05	NaN	NaN	1	2.46429	195	0.14997	5	2.76807	68	0.11995	NaN	4.13783289	GLM
IAIN	RACB75_rep_c110214_250	2	29480370	16.4329	7.26E-05	0.379	0.0672	16.4329	7.26E-05	NaN	NaN	1	2.46429	195	0.14997	5	2.76807	68	0.11995	NaN	4.13783289	GLM
IAIN	850007630.51	14	54376842	16.03225	8.85E-05	0.424	0.05591	16.03225	8.85E-05	NaN	NaN	1	2.43884	195	0.15027	5	2.75698	62	0.12981	NaN	4.05518924	GLM
IAIN	kukl_c3285_145	1	33214958	16.0764	8.95E-05	0.424	0.05583	16.0764	8.95E-05	NaN	NaN	1	2.45542	195	0.15027	5	2.75629	38	0.18424	NaN	4.04678291	GLM
IAIN	Tdum_conf16089_268	2	30113339	15.37097	1.22E-04	0.531	0.05377	15.37097	1.22E-04	NaN	NaN	1	2.31674	195	0.15072	5	2.73856	71	0.11525	NaN	3.91271569	GLM
IAIN	Tdum_conf16089_255	2	30113352	15.37097	1.22E-04	0.531	0.05377	15.37097	1.22E-04	NaN	NaN	1	2.31674	195	0.15072	5	2.73856	71	0.11525	NaN	3.91271569	GLM
IAIN	wrnp_conf288_253	2	29438969	15.2668	1.27E-04	0.545	0.0535	15.2668	1.27E-04	NaN	NaN	1	2.30498	195	0.15078	5	2.7362	69	0.11556	NaN	3.89479684	GLM
IAIN	wrnp_BE471098_To_2_5	2	29439037	15.2668	1.27E-04	0.545	0.0535	15.2668	1.27E-04	NaN	NaN	1	2.30498	195	0.15078	5	2.7362	69	0.11556	NaN	3.89479684	GLM
IAIN	RACB75_c32365_521	2	29463334	15.2668	1.27E-04	0.545	0.0535	15.2668	1.27E-04	NaN	NaN	1	2.30498	195	0.15078	5	2.7362	69	0.11556	NaN	3.89479684	GLM
IAIN	850002924.51	3	56692479	15.27541	1.28E-04	0.546	0.05346	15.27541	1.28E-04	NaN	NaN	1	2.30338	195	0.15079	5	2.73988	20	0.19422	NaN	3.89238307	GLM
IAIN	Tdum_conf10268_1000	10	55539994	15.27972	1.31E-04	0.55	0.05334	15.27972	1.31E-04	NaN	NaN	1	2.29814	195	0.15062	5	2.73484	92	0.12298	NaN	3.88438688	GLM
IAIN	RACB75_rep_c13991_186	2	29387766	15.1924	1.33E-04	0.555	0.05319	15.1924	1.33E-04	NaN	NaN	1	2.2918	195	0.15085	5	2.73357	70	0.11538	NaN	3.874713947	GLM
IAIN	Tdum_conf13991_264	2	29387860	15.1924	1.33E-04	0.555	0.05319	15.1924	1.33E-04	NaN	NaN	1	2.2918	195	0.15085	5	2.73357	70	0.11538	NaN	3.874713947	GLM
IAIN	Tdum_conf13991_404	2	29387884	15.1924	1.33E-04	0.555	0.05319	15.1924	1.33E-04	NaN	NaN	1	2.2918	195	0.15085	5	2.73357	70	0.11538	NaN	3.874713947	GLM
IAIN	TAD0184255	2	29388204	15.1924	1.33E-04	0.555	0.05319	15.1924	1.33E-04	NaN	NaN	1	2.2918	195	0.15085	5	2.73357	70	0.11538	NaN	3.874713947	GLM
IAIN	Tdum_conf1391_480	2	29789225	15.1924	1.33E-04	0.555	0.05319	15.1924	1.33E-04	NaN	NaN	1	2.2918	195	0.15085	5	2.73357	70	0.11538	NaN	3.874713947	GLM
IAIN	Excilburb_c2028_135	2	29789361	15.1924	1.33E-04	0.555	0.05319	15.1924	1.33E-04	NaN	NaN	1	2.2918	195	0.15085	5	2.73357	70	0.11538	NaN	3.874713947	GLM
IAIN	Tdum_conf12289_1857	2	29863602	15.1924	1.33E-04	0.555	0.05319	15.1924	1.33E-04	NaN	NaN	1	2.2918	195	0.15085	5	2.73357	70	0.11538	NaN	3.874713947	GLM
IAIN	Tdum_conf12289_936	2	29864140	15.1924	1.33E-04	0.555	0.05319	15.1924	1.33E-04	NaN	NaN	1	2.2918	195	0.15085	5	2.73357	70	0.11538	NaN	3.874713947	GLM
IAIN	tpb002316_800	2	29997633	15.1924	1.33E-04	0.555	0.05319	15.1924	1.33E-04	NaN	NaN	1	2.2918	195	0.15085	5	2.73357	70	0.11538	NaN	3.874713947	GLM
IAIN	tpb002316_1177	2	29997823	15.1924	1.33E-04	0.555	0.05319	15.1924	1.33E-04	NaN	NaN	1	2.2918	195	0.15085	5	2.73357	70	0.11538	NaN	3.874713947	GLM
IAIN	Tdum_conf11660_338	2	29396669	15.1027	1.40E-04	0.571	0.0529	15.1027	1.40E-04	NaN	NaN	1	2.27201	195	0.15092	5	2.73101	77	0.11238	NaN	3.85523939	GLM
IAIN	IAAV1142	1	29429113	15.0301	1.46E-04	0.586	0.05258	15.0301	1.46E-04	NaN	NaN	1	2.2652	195	0.15096	5	2.72831	63	0.12847	NaN	3.83666527	GLM
IAIN	kukl_c1008_342	2	29707597	14.4585	1.92E-04	0.672	0.0508	14.4585	1.92E-04	NaN	NaN	1	2.1887	195	0.15138	5	2.71295	67	0.11364	NaN	3.71789372	GLM
IAIN	TAD015031009	9	41826820	14.25414	2.12E-04	0.696	0.05031	14.25414	2.12E-04	NaN	NaN	1	2.19988	195	0.15153	5	2.70718	14	0.24412	NaN	3.67807562	GLM
IAIN	AC01339	2	30708704	14.08017	2.31E-04	0.726	0.04956	14.08017	2.31E-04	NaN	NaN	1	2.15359	195	0.15165	5	2.70227	78	0.1085	NaN	3.63338002	GLM
IAIN	Ra_c1195_200	2	29509533	13.92804	2.49E-04	0.747	0.04906	13.92804	2.49E-04	NaN	NaN	1	2.11376	195	0.15176	5	2.69794	80	0.11216	NaN	3.60526272	GLM
IAIN	IAAV561	2	29532694	13.92804	2.49E-04	0.747	0.04906	13.92804	2.49E-04	NaN	NaN	1	2.11376	195	0.15176	5	2.69794	80	0.11216	NaN	3.60526272	GLM
IAIN	IAAV569	2	29560272	13.92804	2.49E-04	0.747	0.04906	13.92804	2.49E-04	NaN	NaN	1	2.11376	195	0.15176	5	2.69794	80	0.11216	NaN	3.60526272	GLM
IAIN	Excilburb_c13058_241	1	33214139	13.7645	2.70E-04	0.767	0.04853	13.7645	2.70E-04	NaN	NaN	1	2.09099	195	0.15188	5	2.69341	43	0.16536	NaN	3.58974151	GLM
IAIN	wrnp_Ru_c3687_11197393	4	67565004	13.48337	3.10E-04	0.803	0.0476	13.48337	3.10E-04	NaN	NaN	1	2.05092	195	0.15208	5	2.68539	74	0.13847	NaN	3.50902225	GLM
IAIN	wrnp_BE517718_To_2_1	10	48749254	13.4306	3.18E-04	0.81	0.04744	13.4306	3.18E-04	NaN	NaN	1	2.0439	195	0.15212	5	2.68399	43	0.16155	NaN	3.49734072	GLM
IAIN	Tdum_conf10968_545	10	55657437	13.2598	3.36E-04	0.827	0.04708	13.2598	3.36E-04	NaN	NaN	1	2.02822	195	0.1522	5	2.68035	79	0.11036	NaN	3.47494904	GLM
IAIN	JD_c3834_381	2	309195474	13.28113	3.44E-04	0.833	0.04693	13.28113	3.44E-04	NaN	NaN	1	2.02184	195	0.15223	5	2.67958	67	0.10985	NaN	3.46376562	GLM
IAIN	Tdum_conf16346_108	2	30203803	13.28113	3.44E-04	0.833	0.04693	13.28113	3.44E-04	NaN	NaN	1	2.02184	195	0.15223	5	2.67958	67	0.10985	NaN	3.46376562	GLM
IAIN	Tdum_conf17497_570	2	30273470	13.28113	3.44E-04	0.833	0.04693	13.28113	3.44E-04	NaN	NaN	1	2.02184	195	0.15223	5	2.67958	67	0.10985	NaN	3.46376562	GLM
IAIN	wrnp_Ec_c14_29750	2	30591692	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	IFL_Conf1354_484	2	30790179	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	Tdum_conf13120_380	2	30790279	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	IFL_Conf1354_403	2	30790338	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	Tdum_conf13120_201	2	30790458	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	Excilburb_c482_30	2	30831120	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	RACB75_c16077_54	2	30831846	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	RACB75_rep_c117462_124	2	30832122	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	850002642.51	2	30832619	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	RACB75_c20420_57	2	30921907	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	BobWhite_c7333_76	2	30980280	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195	0.15225	5	2.67878	94	0.1047	NaN	3.45779994	GLM
IAIN	IFL_Conf12784_641	2	309808134	13.2538	3.49E-04	0.836	0.04684	13.2538	3.49E-04	NaN	NaN	1	2.01784	195								

Table S3.10.2: TASSEL5 results (MLM).

Trail	Marker	Chr	Pos	df	F	p	add_effec1	add_F	add_p	dom_effec1	dom_F	dom_p	errord	MarkerR2	Genetic Var	Residual Var	2xLikelihood		
Item	Name			0	NaN	NaN	NaN	NaN	NaN	NaN	NaN	NaN	197	NaN	0.04571	0.09808	185.93701	MLM	
Item	B00000494_51	14	54489412	1	1414518	2.24E-04	NaN	NaN	NaN	NaN	NaN	NaN	196	0.065	0.03839	0.08603	173.06281	3.65037846	MLM
Item	B00018579_51	14	54519872	1	1381716	2.63E-04	NaN	NaN	NaN	NaN	NaN	NaN	196	0.06358	0.03829	0.08637	173.38201	3.579497615	MLM
Item	wmp_CAP11_c1761_93804	13	98514292	1	1372181	2.76E-04	NaN	NaN	NaN	NaN	NaN	NaN	196	0.0638	0.03575	0.07023	174.02944	3.55912239	MLM
Item	K45_c15985_145	1	33014958	1	1253891	4.99E-04	NaN	NaN	NaN	NaN	NaN	NaN	196	0.05783	0.03447	0.09414	174.89412	3.301936138	MLM
Item	wmp_CARL_c194_818139	7	56359589	1	123001	5.63E-04	NaN	NaN	NaN	NaN	NaN	NaN	196	0.05666	0.03014	0.09742	175.29391	3.220163256	MLM
Item	TA0015231009	9	418288210	1	1173801	7.47E-04	NaN	NaN	NaN	NaN	NaN	NaN	196	0.05457	0.03886	0.08903	175.35398	3.126838849	MLM

Table S3.11: list of genes underlying QTNs.

Gene stable ID	Gene description	Corresponding QTN
TRITD7Av1G261100	GDSL esterase/lipase	BS00021957_51
TRITD7Av1G261110	Myrcene synthase, chloroplastic	BS00021957_51
TRITD7Av1G261120	GDSL esterase/lipase	BS00021957_51
TRITD7Av1G261130	Myrcene synthase, chloroplastic	BS00021957_51
TRITD7Av1G261140	GDSL esterase/lipase	BS00021957_51
TRITD7Av1G261150	Myrcene synthase, chloroplastic	BS00021957_51
TRITD7Av1G261200	40S ribosomal protein S25, putative	BS00021957_51
TRITD7Av1G261250	GDSL esterase/lipase	BS00021957_51
TRITD7Av1G261260	GDSL esterase/lipase	BS00021957_51
TRITD7Av1G261290	GDSL esterase/lipase	BS00021957_51
TRITD7Av1G261300	GDSL esterase/lipase G	BS00021957_51
TRITD7Av1G261330	Histone H2A	BS00021957_51
TRITD7Av1G261380	40S ribosomal protein S27	BS00021957_51
TRITD7Av1G261390	Fatty acid hydroxylase superfamily	BS00021957_51
TRITD7Av1G261420	decapping 5-like protein G	BS00021957_51
TRITD7Av1G261430	RING/U-box superfamily protein	BS00021957_51
TRITD7Av1G261520	Phospholipase A2 G	BS00021957_51
TRITD7Av1G261540		BS00021957_51
TRITD7Av1G261590	Two-component response regulator ARR12 G	BS00021957_51
TRITD7Av1G261600	Aspartic proteinase nepenthesin-1	BS00021957_51
TRITD7Av1G261660	Eukaryotic aspartyl protease family protein	BS00021957_51
TRITD7Av1G261740	Receptor-like kinase	BS00021957_51
TRITD7Av1G261750	Aspartic proteinase nepenthesin-1	BS00021957_51
TRITD7Av1G261840	Heptahelical transmembrane protein 2	BS00021957_51
TRITD7Av1G261880	Plant/T31B5-30 protein	BS00021957_51
TRITD7Av1G261890	Bidirectional sugar transporter SWEET	BS00021957_51
TRITD7Av1G261980	Alkaline alpha-galactosidase seed imbibition protein	BS00021957_51
TRITD7Av1G262010	Cytochrome oxidase assembly 3, mitochondrial G	BS00021957_51
TRITD7Av1G262020	50S ribosomal protein L2, putative	BS00021957_51
TRITD7Av1G262090	serine-rich protein-like protein G	BS00021957_51
TRITD7Av1G262110	DNA-directed RNA polymerase	BS00021957_51
TRITD7Av1G262200	Low temperature and salt responsive protein	BS00021957_51
TRITD7Av1G262230	Patatin	BS00021957_51
TRITD7Av1G262240	Patatin	BS00021957_51
TRITD7Av1G262250	Patatin G	BS00021957_51
TRITD7Av1G262270	Peptide transporter	BS00021957_51
TRITD7Av1G262300	Low temperature and salt responsive protein	BS00021957_51
TRITD7Av1G262310	Zinc finger C-x8-C-x5-C-x3-H type family protein TE?	BS00021957_51
TRITD7Av1G262370	Patatin	BS00021957_51
TRITD7Av1G262380	Beta-catenin-like protein 1	BS00021957_51
TRITD7Av1G262390	BTB/POZ domain protein, putative (DUF177)	BS00021957_51
TRITD7Av1G262400	fimbrin 1 G	BS00021957_51
TRITD7Av1G262430	Ribonucleoside-diphosphate reductase G	BS00021957_51
TRITD7Av1G262490	Protein phosphatase 2c, putative	BS00021957_51
TRITD7Av1G262500	Calmodulin	BS00021957_51

TRITD7Av1G262530	Zinc finger RNA-binding protein	BS00021957_51
TRITD7Av1G262550	Cytochrome P450 family protein, expressed	BS00021957_51
TRITD7Av1G262570	Dihydroflavonol-4-reductase	BS00021957_51
TRITD7Av1G262670	F-box family protein	BS00021957_51
TRITD7Av1G262700	Histone-lysine N-methyltransferase	BS00021957_51
TRITD7Av1G262770	Chaperone protein dnaJ	BS00021957_51
TRITD7Av1G262790	Chaperone protein dnaJ	BS00021957_51
TRITD7Av1G262810	Lectin	BS00021957_51
TRITD7Av1G262820	Phospholipase D Z G	BS00021957_51
TRITD7Av1G262830	Ubiquitin, putative	BS00021957_51
TRITD7Av1G262900	Succinate dehydrogenase subunit 8A, mitochondrial G	BS00021957_51
TRITD7Av1G262910	Magnesium transporter MRS2-like protein G	BS00021957_51
TRITD7Av1G262920	Chaperone protein dnaJ 8 G	BS00021957_51
TRITD7Av1G262930	Lectin	BS00021957_51
TRITD7Av1G262940	BTB/POZ domain containing protein	BS00021957_51
TRITD7Av1G262950	BTB/POZ domain containing protein	BS00021957_51
TRITD7Av1G262960	Histone-lysine N-methyltransferase	BS00021957_51
TRITD7Av1G262980	F-box family protein	BS00021957_51
TRITD7Av1G263030	Protein kinase	BS00021957_51
TRITD7Av1G263050	Protein kinase	BS00021957_51
TRITD7Av1G263130	Protein kinase	BS00021957_51
TRITD7Av1G263140	Protein kinase	BS00021957_51
TRITD7Av1G263160	Protein kinase	BS00021957_51
TRITD7Av1G263210	Protein kinase	BS00021957_51
TRITD7Av1G263230	Carboxypeptidase	BS00021957_51
TRITD7Av1G263240	Transcription elongation factor (TFIIIS) family protein, putative	BS00021957_51
TRITD7Av1G263250	DNA-3-methyladenine glycosylase, putative	BS00021957_51
TRITD7Av1G263720	Cytochrome b561 and DOMON domain-containing protein	BS00021957_51
TRITD7Av1G263810	Leucine-rich repeat receptor-like protein kinase family protein	BS00021957_51
TRITD7Av1G263820	Wd40-repeat-like protein	BS00021957_51
TRITD7Av1G263880	Citrate-binding protein	BS00021957_51
TRITD7Av1G263900	Citrate-binding protein	BS00021957_51
TRITD7Av1G263950	N-alpha-acetyltransferase	BS00021957_51
TRITD7Av1G263980	nuclear pore anchor G	BS00021957_51
TRITD7Av1G264070	LEUNIG-like protein G	BS00021957_51
TRITD7Av1G264080	Invertase/pectin methyltransferase inhibitor family protein	BS00021957_51
TRITD7Av1G264100	LEUNIG-like protein G	BS00021957_51
TRITD7Av1G264160	receptor kinase 1	BS00021957_51
TRITD7Av1G264170	Werner Syndrome-like exonuclease	BS00021957_51
TRITD7Av1G264200	Werner Syndrome-like exonuclease	BS00021957_51
TRITD7Av1G264230	Disease resistance protein RPM1	BS00021957_51
TRITD7Av1G264400	F-box domain containing protein	BS00021957_51
TRITD7Av1G264430	F-box domain containing protein TE?	BS00021957_51
TRITD7Av1G264490	WRKY transcription factor	BS00021957_51
TRITD7Av1G264530	WRKY transcription factor	BS00021957_51
TRITD7Av1G264590	Protein POLAR LOCALIZATION DURING ASYMMETRIC DIVISION AND REDISTRIBUTION G	BS00021957_51
TRITD7Av1G264670	Aldehyde oxidase	BS00021957_51
TRITD7Av1G264700	Glycerol-3-phosphate acyltransferase	BS00021957_51
TRITD7Av1G264740	FBD, F-box and Leucine Rich Repeat domains containing protein	BS00021957_51
TRITD7Av1G264760	Lysosomal Pro-X carboxypeptidase	BS00021957_51
TRITD7Av1G264770	Lysosomal Pro-X carboxypeptidase	BS00021957_51
TRITD7Av1G264780	Lysosomal Pro-X carboxypeptidase	BS00021957_51
TRITD7Av1G264870	Two-component response regulator	BS00021957_51
TRITD7Av1G264880	Two-component response regulator	BS00021957_51
TRITD7Av1G264900		BS00021957_51
TRITD7Av1G264970	50S ribosomal protein L35	BS00021957_51
TRITD7Av1G264990	disease resistance protein (TIR-NBS-LRR class)	BS00021957_51
TRITD7Av1G265020	Disease resistance protein (NBS-LRR class) family	BS00021957_51
TRITD7Av1G265030	Homogenisate phytyltransferase	BS00021957_51
TRITD7Av1G265050	Plasma membrane ATPase	BS00021957_51

TRITD7Av1G265060	Kinase family protein	BS00021957_51
TRITD7Bv1G225000	Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase	BS00062611_51
TRITD7Bv1G224450	Disease resistance protein (TIR-NBS-LRR class)	BS00062611_51
TRITD7Bv1G224250	Callose synthase-like protein G	BS00062611_51
TRITD7Bv1G225550	receptor kinase 1	BS00062611_51
TRITD7Bv1G224050	Receptor-like protein kinase	BS00062611_51
TRITD7Bv1G225790	Disease resistance protein RPM1	BS00062611_51
TRITD7Bv1G224580	RNAse THREE-like protein 3 G	BS00062611_51
TRITD7Bv1G224750	GDSL esterase/lipase	BS00062611_51
TRITD7Bv1G225770	receptor lectin kinase	BS00062611_51
TRITD7Bv1G226420	Transcription factor	BS00062611_51
TRITD7Bv1G225460	Chalcone synthase	BS00062611_51
TRITD7Bv1G225070	12-oxophytodienoate reductase-like protein	BS00062611_51
TRITD7Bv1G226340	Zinc finger-like protein	BS00062611_51
TRITD7Bv1G224400	Disease resistance protein (NBS-LRR class) family	BS00062611_51
TRITD7Bv1G225140	12-oxophytodienoate reductase	BS00062611_51
TRITD7Bv1G225240	Response regulator	BS00062611_51
TRITD7Bv1G226810	KH domain-containing protein SPIN1 G	BS00062611_51
TRITD7Bv1G225300	Disease resistance protein RPM1	BS00062611_51
TRITD7Bv1G225820	Coatomer subunit beta'-1	BS00062611_51
TRITD7Bv1G224840	AT5G11810-like protein G	BS00062611_51
TRITD7Bv1G224710	Protein MEMO1	BS00062611_51
TRITD7Bv1G226380	UPF0496 protein	BS00062611_51
TRITD7Bv1G224490	Cortactin-binding protein 2 G	BS00062611_51
TRITD7Bv1G225220	GDSL esterase/lipase	BS00062611_51
TRITD7Bv1G226050	Protein kinase family protein	BS00062611_51
TRITD7Bv1G224140	Disease resistance protein	BS00062611_51
TRITD7Bv1G224760	GDSL esterase/lipase	BS00062611_51
TRITD7Bv1G226510	Lateral signaling target protein 2-like protein G	BS00062611_51
TRITD7Bv1G224640	GDSL esterase/lipase	BS00062611_51
TRITD7Bv1G225360	Receptor-like protein kinase	BS00062611_51
TRITD7Bv1G224520	Disease resistance protein (NBS-LRR class) family	BS00062611_51
TRITD7Bv1G225420	Reticulon-like protein	BS00062611_51
TRITD7Bv1G225810	Disease resistance protein RPM1	BS00062611_51
TRITD7Bv1G224660	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family G	BS00062611_51
TRITD7Bv1G226530	DUF616 protein	BS00062611_51
TRITD7Bv1G224460	Pm3-like disease resistance protein	BS00062611_51
TRITD7Bv1G224090	Cytochrome P450	BS00062611_51
TRITD7Bv1G226360	Zinc finger family protein	BS00062611_51
TRITD7Bv1G224480	Pm3-like disease resistance protein	BS00062611_51
TRITD7Bv1G225440	receptor kinase 1	BS00062611_51
TRITD7Bv1G225610	lectin-receptor kinase	BS00062611_51
TRITD7Bv1G225210	Protein phosphatase 2C	BS00062611_51
TRITD7Bv1G224730		BS00062611_51
TRITD7Bv1G224500	Disease resistance protein (NBS-LRR class) family	BS00062611_51
TRITD7Bv1G226330	Zinc finger-like protein	BS00062611_51
TRITD7Bv1G224060	Bidirectional sugar transporter SWEET	BS00062611_51
TRITD7Bv1G225480	Receptor-like protein kinase	BS00062611_51
TRITD7Bv1G224370	Disease resistance protein	BS00062611_51
TRITD7Bv1G224220	Disease resistance protein	BS00062611_51
TRITD7Bv1G225330	receptor kinase 1	BS00062611_51
TRITD7Bv1G226540	DDB1-and CUL4-associated factor	BS00062611_51
TRITD7Bv1G227150	RING/FYVE/PHD zinc finger superfamily protein	BS00062611_51
TRITD7Bv1G226240	Zinc finger family protein	BS00062611_51
TRITD7Bv1G226410	Blue copper protein	BS00062611_51
TRITD7Bv1G224170		BS00062611_51
TRITD7Bv1G225950	Wall-associated receptor kinase 2	BS00062611_51
TRITD7Bv1G226000	Axonemal dynein light intermediate polypeptide 1 G	BS00062611_51
TRITD7Bv1G225400	Hydroxysteroid dehydrogenase, putative	BS00062611_51
TRITD7Bv1G224130	WD40 repeat-like protein	BS00062611_51

TRITD7Bv1G225010	DNA topoisomerase G	BS00062611_51
TRITD7Bv1G225630	protein kinase family protein	BS00062611_51
TRITD7Bv1G225060	Eukaryotic aspartyl protease family protein	BS00062611_51
TRITD7Bv1G224590	GD5L esterase/lipase	BS00062611_51
TRITD7Bv1G226500	Zinc finger FYVE domain-containing protein 1	BS00062611_51
TRITD7Bv1G224770	GD5L esterase/lipase	BS00062611_51
TRITD7Bv1G224230	BTB/POZ domain containing protein	BS00062611_51
TRITD7Bv1G226120	Receptor-like kinase	BS00062611_51
TRITD7Bv1G225430	50S ribosomal protein L9	BS00062611_51
TRITD7Bv1G226260	Zinc finger family protein	BS00062611_51
TRITD7Bv1G225390	NBS-LRR disease resistance protein-like	BS00062611_51
TRITD7Bv1G225600	Heavy metal transport/detoxification superfamily protein, putative	BS00062611_51
TRITD7Bv1G225350	Polyamine oxidase	BS00062611_51
TRITD7Bv1G224040	RNA polymerase II transcription mediator G	BS00062611_51
TRITD7Bv1G225910	Disease resistance protein	BS00062611_51
TRITD7Bv1G227030	Serine/threonine-protein kinase	BS00062611_51
TRITD7Bv1G224630	Werner Syndrome-like exonuclease	BS00062611_51
TRITD7Bv1G225660	F-box domain containing protein TE?	BS00062611_51
TRITD7Bv1G224270	RING/U-box superfamily protein	BS00062611_51
TRITD7Bv1G226550	RNA-binding protein	BS00062611_51
TRITD7Bv1G224350	F-box domain containing protein	BS00062611_51
TRITD7Bv1G225560	NADH-ubiquinone oxidoreductase chain 3	BS00062611_51
TRITD7Bv1G224160	NBS-LRR-like resistance protein	BS00062611_51
TRITD7Bv1G224940	Cytokinin oxidase/dehydrogenase	BS00062611_51
TRITD7Bv1G224720	ARM repeat superfamily protein	BS00062611_51
TRITD7Bv1G224120	Pseudouridine synthase	BS00062611_51
TRITD7Bv1G224870	GD5L esterase/lipase	BS00062611_51
TRITD7Bv1G225990	Glyceraldehyde-3-phosphate dehydrogenase	BS00062611_51
TRITD7Bv1G224470	disease resistance family protein / LRR family protein	BS00062611_51
TRITD7Bv1G227050	Basic helix-loop-helix transcription factor	BS00062611_51
TRITD7Bv1G225130	12-oxophytodienoate reductase-like protein	BS00062611_51
TRITD7Bv1G225580	Cytochrome c oxidase subunit 2	BS00062611_51
TRITD7Bv1G225570	30S ribosomal protein S12	BS00062611_51
TRITD7Bv1G224560	Premnaspirodiene oxygenase	BS00062611_51
TRITD7Bv1G225370	Disease resistance protein	BS00062611_51
TRITD7Bv1G224830	GD5L esterase/lipase	BS00062611_51
TRITD7Bv1G226110	Disease resistance protein RPM1	BS00062611_51
TRITD7Bv1G225760	Coatomer subunit beta'-1	BS00062611_51
TRITD7Bv1G170830	Gamma-aminobutyric acid type B receptor subunit 1 G	BS00108573_51
TRITD7Bv1G170910	Flavonoid 3'-hydroxylase	BS00108573_51
TRITD7Bv1G170930	Protein DETOXIFICATION	BS00108573_51
TRITD7Bv1G170940	Upf0496 protein	BS00108573_51
TRITD7Bv1G171020	Glycosyltransferase	BS00108573_51
TRITD7Bv1G171030	Homeodomain-like superfamily protein G	BS00108573_51
TRITD7Bv1G171080	Peptide transporter, putative	BS00108573_51
TRITD7Bv1G171140	Serine/threonine-protein kinase	BS00108573_51
TRITD7Bv1G171300	12-oxophytodienoate reductase	BS00108573_51
TRITD7Bv1G171310	Deoxyuridine 5'-triphosphate nucleotidohydrolase	BS00108573_51
TRITD7Bv1G171340	Glycosyltransferase	BS00108573_51
TRITD7Bv1G171360	60S ribosomal protein L35a-like protein	BS00108573_51
TRITD7Bv1G171390	Glycosyltransferase	BS00108573_51
TRITD7Bv1G171480	FBD-associated F-box protein	BS00108573_51
TRITD7Bv1G171550	Peptidyl-prolyl cis-trans isomerase	BS00108573_51
TRITD7Bv1G171560	Zinc-transporting ATPase	BS00108573_51
TRITD7Bv1G171780	ATP-dependent RNA helicase	BS00108573_51
TRITD7Bv1G171790	UDP-sugar pyrophosphorylase 1	BS00108573_51
TRITD7Bv1G171800	UPF0214 protein YfeW	BS00108573_51
TRITD7Bv1G171980	E3 ubiquitin-protein ligase	BS00108573_51
TRITD7Bv1G172210	60S acidic ribosomal protein P3	BS00108573_51
TRITD7Bv1G172240	Beta-carotene isomerase D27, chloroplastic	BS00108573_51

TRITD7Bv1G172250	Pentatricopeptide repeat-containing protein	BS00108573_51
TRITD7Bv1G172280	RELA/SPOT G	BS00108573_51
TRITD7Bv1G172300	Chaperone protein DnaJ G	BS00108573_51
TRITD7Bv1G172350	HKT23 transporter	BS00108573_51
TRITD7Bv1G172380	Sodium transporter	BS00108573_51
TRITD7Bv1G172390	ArfGap/RecO-like zinc finger domain-containing protein TE?	BS00108573_51
TRITD7Bv1G172420	HKT23 transporter	BS00108573_51
TRITD7Bv1G172530	SAUR-like auxin-responsive family protein	BS00108573_51
TRITD7Bv1G172540	Protein EFR3-like protein	BS00108573_51
TRITD7Bv1G172590	L-lactate dehydrogenase	BS00108573_51
TRITD7Bv1G172620	Ankyrin repeat family protein, putative	BS00108573_51
TRITD7Bv1G172630	Ankyrin repeat family protein, putative	BS00108573_51
TRITD7Bv1G172670	14 kDa proline-rich protein DC2.15	BS00108573_51
TRITD7Bv1G172680	L-lactate dehydrogenase	BS00108573_51
TRITD7Bv1G172690	Methyl-CpG-binding domain protein	BS00108573_51
TRITD7Bv1G172710	F-box family protein	BS00108573_51
TRITD7Bv1G172730	O-glucosyltransferase rumi like	BS00108573_51
TRITD7Bv1G172890	carboxyl-terminal peptidase (DUF239)	BS00108573_51
TRITD7Bv1G172950	Galactokinase	BS00108573_51
TRITD7Bv1G172970	TCP family transcription factor 4 G	BS00108573_51
TRITD7Bv1G173020	Type 1 membrane protein-like G	BS00108573_51
TRITD7Bv1G173030	Disease resistance protein RPM1	BS00108573_51
TRITD7Bv1G173200	Auxin response factor	BS00108573_51
TRITD7Bv1G173210	Protein AIG2	BS00108573_51
TRITD7Bv1G173220	Protein MID1-COMPLEMENTING ACTIVITY 1 G	BS00108573_51
TRITD7Bv1G173260	Protein MID1-COMPLEMENTING ACTIVITY 1 G	BS00108573_51
TRITD7Bv1G173290	3-phosphoinositide-dependent protein kinase-1-like G	BS00108573_51
TRITD7Bv1G173320	26S proteasome regulatory subunit	BS00108573_51
TRITD7Bv1G173450	MYB transcription factor	BS00108573_51
TRITD7Bv1G173680		BS00108573_51
TRITD7Bv1G173690	GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase	BS00108573_51
TRITD7Bv1G173700	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	BS00108573_51
TRITD6Av1G053650	Bidirectional sugar transporter SWEET	CAP12_rep_c4638_80
TRITD6Av1G053800	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	CAP12_rep_c4638_80
TRITD6Av1G053830	Autophagy-related protein 11	CAP12_rep_c4638_80
TRITD6Av1G054030	Plant/T31B5-30 protein	CAP12_rep_c4638_80
TRITD6Av1G054050	Heptahelical transmembrane protein 2	CAP12_rep_c4638_80
TRITD6Av1G054180	Tubby-like F-box protein	CAP12_rep_c4638_80
TRITD6Av1G054190	Ubiquitin family protein	CAP12_rep_c4638_80
TRITD6Av1G054870	GMP synthase [glutamine-hydrolyzing]	CAP12_rep_c4638_80
TRITD6Av1G054920	HD domain-containing protein 2	CAP12_rep_c4638_80
TRITD6Av1G054950	Kinase	CAP12_rep_c4638_80
TRITD6Av1G055130	Fatty acid hydroxylase superfamily	CAP12_rep_c4638_80
TRITD6Av1G055170	Flavin-containing monooxygenase	CAP12_rep_c4638_80
TRITD6Av1G055190	Basic helix-loop-helix transcription factor	CAP12_rep_c4638_80
TRITD6Av1G055370	DNA-directed RNA polymerase subunit beta	CAP12_rep_c4638_80
TRITD6Av1G055540	Protein CYPRO4	CAP12_rep_c4638_80
TRITD6Av1G055560	Protein transport protein Sec61 subunit gamma	CAP12_rep_c4638_80
TRITD6Av1G055660	Flavin-containing monooxygenase	CAP12_rep_c4638_80
TRITD6Av1G055770	Glutamine dumper, putative G	CAP12_rep_c4638_80
TRITD6Av1G055780	CONSTANS-like protein	CAP12_rep_c4638_80
TRITD6Av1G055860	Non-specific serine/threonine protein kinase	CAP12_rep_c4638_80
TRITD6Av1G055970	Glycerol kinase	CAP12_rep_c4638_80
TRITD6Av1G056010	Calmodulin binding protein	CAP12_rep_c4638_80
TRITD6Av1G056030	CASP-like protein	CAP12_rep_c4638_80
TRITD6Av1G056150	Tryptophan--tRNA ligase G	CAP12_rep_c4638_80
TRITD6Av1G056180	F-box plant-like protein, putative	CAP12_rep_c4638_80
TRITD6Av1G056270	4-coumarate coenzyme A ligase	CAP12_rep_c4638_80
TRITD6Av1G056280	4CL G	CAP12_rep_c4638_80
TRITD6Av1G056570	Alpha-1,4-glucan-protein synthase [UDP-forming]	CAP12_rep_c4638_80

TRITD6Av1G056630	Squamosa promoter binding protein-like protein	CAP12_rep_c4638_80
TRITD6Av1G056790	Fasciclin-like arabinogalactan protein	CAP12_rep_c4638_80
TRITD6Av1G056990	CAI-1 autoinducer sensor kinase/phosphatase cqsS isoform 2	CAP12_rep_c4638_80
TRITD6Av1G057010	Aldose 1-epimerase family protein	CAP12_rep_c4638_80
TRITD6Av1G057020	Centrosomal protein of 76 kDa G	CAP12_rep_c4638_80
TRITD6Av1G057030	Glucose-methanol-choline (GMC) oxidoreductase family protein G	CAP12_rep_c4638_80
TRITD6Av1G057110	Calcium-transporting ATPase	CAP12_rep_c4638_80
TRITD4Bv1G181440	Zinc finger protein	GENE-4008_418
TRITD4Bv1G178880	Aquaporin	GENE-4008_419
TRITD4Bv1G182330	Zinc finger-like protein	GENE-4008_419
TRITD4Bv1G182210	auxin response factor 1 G	GENE-4008_419
TRITD4Bv1G180690	Sulfotransferase	GENE-4008_419
TRITD4Bv1G179100	HEAT repeat-containing 7A-like protein	GENE-4008_419
TRITD4Bv1G179810	Cortical cell-delineating protein	GENE-4008_419
TRITD4Bv1G179030	VQ motif family protein, expressed	GENE-4008_419
TRITD4Bv1G179980	Methyltransferase-like protein 1	GENE-4008_419
TRITD4Bv1G182280	Chaperone protein dnaJ	GENE-4008_419
TRITD4Bv1G179050	Pentatricopeptide repeat-containing protein	GENE-4008_419
TRITD4Bv1G180060	UPF0496 protein	GENE-4008_419
TRITD4Bv1G178920	Pheophorbide a oxygenase, chloroplastic	GENE-4008_419
TRITD4Bv1G180550	Zinc finger protein	GENE-4008_419
TRITD4Bv1G180790	LOB domain-containing protein, putative	GENE-4008_419
TRITD4Bv1G180870	HR-like lesion-inducing protein-related protein	GENE-4008_419
TRITD4Bv1G180270	Phosphate import ATP-binding protein PstB	GENE-4008_419
TRITD4Bv1G179180	Receptor kinase-like protein	GENE-4008_419
TRITD4Bv1G181200	Phosphate transporter protein	GENE-4008_419
TRITD4Bv1G180900	WAT1-related protein	GENE-4008_419
TRITD4Bv1G182270	C-terminal binding protein AN G	GENE-4008_419
TRITD4Bv1G179190	BTB/POZ domain containing protein, expressed	GENE-4008_419
TRITD4Bv1G178990	VQ motif family protein, expressed	GENE-4008_419
TRITD4Bv1G180320	Protein kinase	GENE-4008_419
TRITD4Bv1G181790	D111/G-patch domain-containing protein, putative, expressed	GENE-4008_419
TRITD4Bv1G180910	Protein KINESIN LIGHT CHAIN-RELATED 3	GENE-4008_419
TRITD4Bv1G181280	Phosphate transporter protein	GENE-4008_419
TRITD4Bv1G180150	Ubiquitin-conjugating enzyme E2	GENE-4008_419
TRITD4Bv1G180050	Phosphoribosylamine-glycine ligase	GENE-4008_419
TRITD4Bv1G181230	Phosphate transporter protein	GENE-4008_419
TRITD4Bv1G181960	Diacylglycerol kinase	GENE-4008_419
TRITD4Bv1G182200	DUF21 domain-containing protein	GENE-4008_419
TRITD4Bv1G179940	receptor kinase 1	GENE-4008_419
TRITD4Bv1G182310	S-adenosyl-L-methionine-dependent methyltransferase domain-containing protein	GENE-4008_419
TRITD4Bv1G181190	Ethylene-responsive transcription factor	GENE-4008_419
TRITD4Bv1G180460	Defensin	GENE-4008_419
TRITD4Bv1G181310	Phosphate transporter protein	GENE-4008_419
TRITD4Bv1G179370	Ethylene-responsive transcription factor	GENE-4008_419
TRITD4Bv1G180290	UPF0503 protein, chloroplastic	GENE-4008_419
TRITD4Bv1G180000	Tubulin-folding cofactor C	GENE-4008_419
TRITD4Bv1G179760	RNA-binding protein	GENE-4008_419
TRITD4Bv1G179270	Cytochrome P450, putative	GENE-4008_419
TRITD4Bv1G182260	Anthranilate phosphoribosyltransferase	GENE-4008_419
TRITD4Bv1G180030	Glycosyltransferase	GENE-4008_419
TRITD4Bv1G181750		GENE-4008_419
TRITD4Bv1G180800	LOB domain-containing protein, putative	GENE-4008_419
TRITD4Bv1G180650	Benzyl alcohol O-benzoyltransferase	GENE-4008_419
TRITD4Bv1G182320	S-adenosyl-L-methionine-dependent methyltransferase domain-containing protein	GENE-4008_419
TRITD4Bv1G179850	Cytokinin riboside 5'-monophosphate phosphoribohydrolase G	GENE-4008_419
TRITD4Bv1G181060	Ring finger protein, putative	GENE-4008_419
TRITD4Bv1G182010	Kinase family protein	GENE-4008_419
TRITD4Bv1G179170	Exocyst complex component, putative	GENE-4008_419
TRITD4Bv1G181480	WD40 repeat-like protein	GENE-4008_419

TRITD4Bv1G182160	disease resistance protein (TIR-NBS-LRR class)	GENE-4008_419
TRITD4Bv1G179540	Divalent ion symporter	GENE-4008_419
TRITD4Bv1G180330	tornado 1 G	GENE-4008_419
TRITD4Bv1G181320	Appr-1-p processing enzyme family protein	GENE-4008_419
TRITD4Bv1G181240	Phosphate transporter protein	GENE-4008_419
TRITD4Bv1G180440	Receptor-like kinase	GENE-4008_419
TRITD4Bv1G182080	DET1-and DDB1-associated protein 1	GENE-4008_419
TRITD4Bv1G181720	Beta-carotene hydroxylase	GENE-4008_419
TRITD4Bv1G179830	Cortical cell-delineating protein	GENE-4008_419
TRITD4Bv1G180140	kinase with tetratricopeptide repeat domain-containing protein G	GENE-4008_419
TRITD4Bv1G179090	NADH-quinone oxidoreductase subunit D	GENE-4008_419
TRITD4Bv1G179260	Cysteine/O-acetylserine efflux protein G	GENE-4008_419
TRITD4Bv1G178980	VQ motif family protein, expressed	GENE-4008_419
TRITD2Bv1G136300	Mis18-binding protein 1	Tdurum_config67516_236
TRITD2Bv1G136340	C2H2 and C2HC zinc fingers superfamily protein TE?	Tdurum_config67516_236
TRITD2Bv1G136350	Protein embryo defective 1674	Tdurum_config67516_236
TRITD2Bv1G136520	Kinesin-like protein	Tdurum_config67516_236
TRITD2Bv1G136640	Carbohydrate-binding X8 domain superfamily protein G	Tdurum_config67516_236
TRITD2Bv1G136700	Bifunctional lysine-specific demethylase and histidyl-hydroxylase NO66 G	Tdurum_config67516_236
TRITD2Bv1G136730	Cysteine proteinase inhibitor	Tdurum_config67516_236
TRITD2Bv1G136870	Exocyst complex component exo70a1	Tdurum_config67516_236
TRITD2Bv1G136960	40S ribosomal protein S8	Tdurum_config67516_236
TRITD2Bv1G137020	U11/U12 small nuclear ribonucleoprotein 48 kDa protein G	Tdurum_config67516_236
TRITD2Bv1G137080	Dead box ATP-dependent RNA helicase, putative	Tdurum_config67516_236
TRITD2Bv1G137210	U-box domain-containing protein	Tdurum_config67516_236
TRITD2Bv1G137280	DnaJ / Sec63 Brl domains-containing protein G	Tdurum_config67516_236
TRITD2Bv1G137300	Protein kinase superfamily protein G	Tdurum_config67516_236
TRITD2Bv1G137350	Peptidylprolyl isomerase	Tdurum_config67516_236
TRITD2Bv1G137360	Kinase family protein	Tdurum_config67516_236
TRITD2Bv1G137400	Pentatricopeptide repeat-containing protein	Tdurum_config67516_236
TRITD2Bv1G137410	Pentatricopeptide repeat-containing protein	Tdurum_config67516_236
TRITD2Bv1G137420	Phospholipid-transporting ATPase	Tdurum_config67516_236
TRITD2Bv1G137500	sodium hydrogen exchanger 4 G	Tdurum_config67516_236
TRITD2Bv1G137540	DNA-directed RNA polymerase subunit beta	Tdurum_config67516_236
TRITD2Bv1G137580	Alcohol dehydrogenase	Tdurum_config67516_236
TRITD2Bv1G137590	L-threonine 3-dehydrogenase	Tdurum_config67516_236
TRITD2Bv1G137770	Alcohol dehydrogenase 3	Tdurum_config67516_236
TRITD2Bv1G137780	Nuclear transport factor 2 family protein with RNA binding domain	Tdurum_config67516_236
TRITD2Bv1G137850	50S ribosomal protein L29	Tdurum_config67516_236
TRITD2Bv1G137860	Protein DETOXIFICATION	Tdurum_config67516_236
TRITD2Bv1G137920	Protein DETOXIFICATION	Tdurum_config67516_236
TRITD2Bv1G137980	U-box domain-containing protein	Tdurum_config67516_236
TRITD2Bv1G138000	magnesium transporter NIPA (DUF803)	Tdurum_config67516_236
TRITD2Bv1G138260	Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein G	Tdurum_config67516_236
TRITD2Bv1G138460	3-oxoacyl-reductase	Tdurum_config67516_236
TRITD2Bv1G138490	Chitinase-like protein	Tdurum_config67516_236
TRITD2Bv1G138550	Eukaryotic translation initiation factor 3 subunit H G	Tdurum_config67516_236
TRITD2Bv1G138610	Protein XR11 G	Tdurum_config67516_236
TRITD2Bv1G138650	NudC domain-containing protein 2	Tdurum_config67516_236
TRITD2Bv1G138830	TBC1 domain family member	Tdurum_config67516_236
TRITD2Bv1G138950	Amino acid transporter, putative	Tdurum_config67516_236

Table S3.12: list of genes underlying BS00108573_51.

Gene stable ID	Gene description	KEGG annotation	KEGG pathway
TRII D7bvIG171300	12-oxophytodienoate reductase	OPR; 12-oxophytodienoic acid reductase [EC:1.3.1.42]	K05894
TRII D7bvIG172670	14 kDa proline-rich protein DC2.15		
TRII D7bvIG173320	26S proteasome regulatory subunit	PSMD14, RPN11, POH1; 26S proteasome regulatory subunit N11	K03030
TRII D7bvIG173290	3-phosphoinositide-dependent protein kinase-1-like G		
TRII D7bvIG172210	60S acidic ribosomal protein P3	RP-LP1, RPLP1; large subunit ribosomal protein LP1	K02946
TRII D7bvIG171360	60S ribosomal protein L35a-like protein	RP-L35Ae, RPL35A; large subunit ribosomal protein L35Ae	K02917
TRII D7bvIG172620	Ankyrin repeat family protein, putative		
TRII D7bvIG172630	Ankyrin repeat family protein, putative		
TRII D7bvIG172390	ArtGap/RecO-like zinc finger domain-containing protein TE3		
TRII D7bvIG171780	ATP-dependent RNA helicase		
TRII D7bvIG173200	Auxin response factor		
TRII D7bvIG172240	Beta-carotene isomerase D27, chloroplastic	DWARF27; beta-carotene isomerase [EC:5.2.1.14]	K17911
TRII D7bvIG172890	carboxyl-terminal peptidase (DUF239)		
TRII D7bvIG172300	Chaperone protein DnaJ G		
TRII D7bvIG171310	Deoxyuridine 5-triphosphate nucleotidohydrolase		
TRII D7bvIG173030	Disease resistance protein RPM1		
TRII D7bvIG173700	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit	oligosaccharyltransferase complex subunit beta	K12670
TRII D7bvIG171980	E3 ubiquitin-protein ligase	SAH1; E3 ubiquitin-protein ligase SAH1 [EC:2.3.2.27]	K04506
TRII D7bvIG172710	F-box family protein		
TRII D7bvIG171480	FBD-associated F-box protein		
TRII D7bvIG170910	Flavonoid 3-hydroxylase	CYP75B1; flavonoid 3-monoxygenase [EC:1.14.14.82]	K05280
TRII D7bvIG172950	Galactokinase	L-arabinokinase [EC:2.7.1.46]	K12446
TRII D7bvIG170830	Gamma-aminobutyric acid type B receptor subunit I G		
TRII D7bvIG171020	Glycosyltransferase		
TRII D7bvIG171340	Glycosyltransferase		
TRII D7bvIG171390	Glycosyltransferase		
TRII D7bvIG173690	GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase	rib8A; 3,4-dihydroxy 2-butanone 4-phosphate synthase / GTP cyclohydrolase II [EC:4.1.99.12 3.5.4.25]	K14652
TRII D7bvIG172350	HKT23 transporter		
TRII D7bvIG172420	HKT23 transporter		
TRII D7bvIG171030	Homeodomain-like superfamily protein G		
TRII D7bvIG172590	L-lactate dehydrogenase	LDH, ldh; L-lactate dehydrogenase [EC:1.1.1.27]	K00016
TRII D7bvIG172680	L-lactate dehydrogenase	LDH, ldh; L-lactate dehydrogenase [EC:1.1.1.27]	K00016
TRII D7bvIG172690	Methyl-CpG-binding domain protein		
TRII D7bvIG173450	MYB transcription factor		
TRII D7bvIG172730	O-glucosyltransferase rumi like		
TRII D7bvIG172250	Pentatricopeptide repeat-containing protein		
TRII D7bvIG171080	Peptide transporter, putative		
TRII D7bvIG171550	Peptidyl-prolyl cis-trans isomerase		
TRII D7bvIG173210	Protein AG2		
TRII D7bvIG170930	Protein DETOXIFICATION		
TRII D7bvIG172540	Protein EFR3-like protein		
TRII D7bvIG173220	Protein MIDI-COMPLEMENTING ACTIVITY 1 G		
TRII D7bvIG173260	Protein MIDI-COMPLEMENTING ACTIVITY 1 G		
TRII D7bvIG172280	RELA/SPOT G		
TRII D7bvIG172530	SAUR-like auxin-responsive family protein	SAUR family protein	K14488
TRII D7bvIG171140	Serine/threonine-protein kinase		
TRII D7bvIG172380	Sodium transporter		
TRII D7bvIG172970	TCP family transcription factor 4 G		
TRII D7bvIG173020	Type 1 membrane protein-like G		
TRII D7bvIG171790	UDP-sugar pyrophosphorylase 1	UDP-sugar pyrophosphorylase [EC:2.7.7.64]	K12447
TRII D7bvIG171800	UPF0214 protein YfeW		
TRII D7bvIG170940	Upf0496 protein		
TRII D7bvIG171560	Zinc-transporting ATPase		
TRII D7bvIG173680	protein coding putative		

Chapter 4. Exploring Variability of Free Asparagine Content in the Grain of Bread Wheat (*Triticum aestivum* L.) Varieties Cultivated in Italy to Reduce Acrylamide-Forming Potential

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Plants 2023, 12, 1349. <https://doi.org/10.3390/plants12061349>

The results presented in this chapter have been published in the article:

Tafuri, A.; Zuccaro, M.; Ravaglia, S.; Pirona, R.; Masci, S.; Sestili, F.; Lafiandra, D.; Ceriotti, A.; Baldoni, E. Exploring Variability of Free Asparagine Content in the Grain of Bread Wheat (*Triticum aestivum* L.) Varieties Cultivated in Italy to Reduce Acrylamide-Forming Potential. *Plants* 2023, 12, 1349. <https://doi.org/10.3390/plants12061349>

Abstract

Acrylamide, a suspected human carcinogen, is generated during food processing at high temperatures in the Maillard reaction, which involves reducing sugars and free asparagine. In wheat derivatives, free asparagine represents a key factor in acrylamide formation. Free asparagine levels in the grain of different wheat genotypes have been investigated in recent studies, but little is known about elite varieties that are cultivated in Italy. Here, we analysed the accumulation of free asparagine in a total of 54 bread wheat cultivars that are relevant for the Italian market. Six field trials in three Italian locations over two years were considered. Wholemeal flours obtained from harvested seeds were analysed using an enzymatic method. Free asparagine content ranged from 0.99 to 2.82 mmol/kg dry matter in the first year, and from 0.55 to 2.84 mmol/kg dry matter in the second year. Considering the 18 genotypes that were present in all the field trials, we evaluated possible environment and genetic influences for this trait. Some cultivars seemed to be highly affected by environment, whereas others showed a relative stability in free asparagine content across years and locations. Finally, we identified two varieties showing the highest free asparagine levels in our analysis, representing potential useful materials for genotype x environment interaction studies. Two other varieties, which were characterized by low amounts of free asparagine in the considered samples, may be useful for the food industry and for future breeding programs aimed to reduce acrylamide-forming potential in bread wheat.

4.1 Introduction

Wheat (*Triticum* spp. L.) is one of the world's most important crops, contributing an estimated 18.6% to global daily calorie intake and 19.8% to global daily protein intake in 2018 (<http://www.fao.org/faostat/en/> (accessed on 7 January 2023)). The world currently produces around 770 mmt of wheat annually [1]. A key reason for the importance of wheat as a staple crop is its ability to be used as the main ingredient in a plethora of products, such as breads, noodles, couscous, and pasta. In Southern Europe, about 5 million hectares were used for wheat cultivation in 2021, with a production of about 22 million tonnes. In 2020, in the same geographic area, per capita consumption of wheat was estimated at about 115 kg (<http://www.fao.org/faostat/en/> (accessed on 7 January 2023)). In Italy, wheat represents an important part of the national cereal market with more than 7 million tonnes produced in 2021, as shown also by a 3.3% increase in production compared to the average of the last 5 years (<https://agridata.ec.europa.eu/extensions/DashboardCereals/CerealsProduction.html> (accessed on 6 December 2022)).

Wheat breeding is principally aimed to elevate yield potential with stability, reduce the requirements for water, fertilizers, and other inputs without affecting productivity, increase the ability of wheat to grow on marginal lands, and protect wheat production from emerging threats of climate change [2]. Today, one of the objectives of wheat breeding is the improvement of its nutritional quality and health value [3,4]. A major issue related to food production that can be faced through specific breeding programs is the reduction of acrylamide, which is generated in a wide range of foods, including wheat derivatives [5,6]. Acrylamide (C_3H_5NO) is a white, odourless, water-soluble molecule and is rapidly absorbed through the skin and from the gastro-intestinal tract. Once metabolised, it produces glycidamide ($C_3H_5NO_2$), which forms adducts with DNA both in vitro and in vivo in human and animal tissues [7]. Acrylamide is classed in group 2a as a probable carcinogenic to humans by the International Agency for Research on Cancer (IARC) [8]. Acrylamide is

generated by Maillard cascade reactions during high-temperature processing of foods (frying, baking, roasting, toasting) [9–12]. These reactions involve free reducing sugars, such as glucose, fructose, and maltose, and free asparagine (fAsn), which gives the carbon skeleton. The fAsn concentration has been identified as the main determinant of acrylamide-forming potential in wheat and other cereals [12–14]. For this reason, a major recommendation in the food industry to reduce acrylamide levels cereal-based products is the use of grain with low fAsn content [15]. Free amino acids generally account for about 5% or less of the total nitrogen content of wheat grain, with fAsn accounting for 10% or less of the total [16]. Consequently, this amino acid was of little scientific interest until the demonstration that it is a precursor of acrylamide during cooking. Only in recent years has an increasing number of studies aimed to compare the level of fAsn in the seeds of different wheat genotypes and to identify the factors influencing this trait. Variability in fAsn accumulation in the grain is high among wheat genotypes [15,17–19]. It is well demonstrated that this trait is highly influenced by environmental factors, such as abiotic and biotic stresses [16], sulphur or nitrogen availability [11,13,20–24], and poor disease control [21,25]. Nevertheless, the accumulation of fAsn in wheat seed is a specific trait showing good heritability [26,27]. In addition, genotype versus environmental (GxE) effects influence fAsn content in wheat seeds [13,15,18,28,29]. Thus, both breeding and genetics may play an important role in selecting and developing cultivars accumulating low and stable fAsn levels across different growing environments, and then to mitigate the acrylamide risk in wheat products. Most of the studies about fAsn in wheat grain have been related to bread wheat (*Triticum aestivum* L.) genotypes that are principally cultivated in the U.S., the UK, Canada, and Australia [15,18]. These studies underlined that many differences in fAsn concentration were due to the growing environment and crop management, although some cultivars may be considered as low asparagine accumulating genotypes for the involvement of genetic factors. Contrastingly, there is little information on fAsn concentration in bread wheat varieties cultivated in Italy. To our knowledge, only Corol and collaborators [18]

included in their study two bread wheat varieties cultivated and commercialized in Italy (i.e., Blasco and Palesio), both classified as low fAsn genotypes. Considering the importance of bread wheat in the Italian agricultural system and food industry, the aim of this study was to perform a preliminary evaluation of fAsn content in the grain of selected elite varieties of bread wheat, relevant for the Italian market, to set the base of a characterization of this genetic material in terms of acrylamide-forming potential. We considered a total of 54 genotypes, that are widely cultivated in Italy, and three growing locations over two years to evaluate possible influences of both environmental and genetic factors. Hence, our analysis allowed for the identification of cultivars showing relatively low and stable fAsn levels. These genotypes may be used in future breeding programs aimed to mitigate the acrylamide risk in wheat derivatives.

4.2. Results and Discussion

4.2.1. Free Asparagine Quantification in Six Field Trials Over Two Years

Field trials of selected bread wheat elite varieties relevant for the Italian market were performed over two years (2018–2019 and 2019–2020) at three locations across Southern, Central, and Northern Italy (Foggia, Grosseto, Voghera, respectively). There were 54 varieties altogether over the two trial seasons: 36 genotypes in 2018–2019 and 36 genotypes in 2019–2020, with 18 varieties being present in both. The genotypes are listed in Table 4.1, grouped according to the Italian bread wheat classification based on chemical and rheological properties [30,31]. Bread wheat is unique for the production of leavened food, bread and biscuits in particular. The classification of different genotypes relies on several physical, chemical, and rheological characteristics. Worldwide, various classification systems are principally based on objective evaluations of gluten proteins. In Italy, bread wheat is classified into four categories, depending on its end use [31]. The specific classes are: (i) FF = improver wheat ("Frumento di Forza" in Italian), varieties suitable for manufacturing highly leavened products with a very resistant gluten network; (ii) FPS = superior bread

making wheat ("Frumento Panificabile Superiore"), varieties with consistent milling and baking performance; (iii) FP = ordinary bread making wheat ("Frumento Panificabile"), varieties with bread-making potential but not suited to all grists; (iv) FB = wheat for biscuits ("Frumento da Biscotto"), soft varieties used for biscuits, breakfast cereals, cakes, and similar products. The variety list for 2018–2019 comprised six FF varieties, five FPS varieties, twenty FP varieties, and five FB varieties (Table 4.1). The variety list for 2019–2020 comprised six FF varieties, nine FPS varieties, eighteen FP varieties, and three FB varieties (Table 4.1). A recent study underlined that there are no significant differences in free asparagine levels among different classes related to rheological characteristics [15]. Nevertheless, it may be useful to indicate the classification of the analysed genotypes to help farmers and the food industry in decision making. Wholemeal flour was produced from the grain harvested from the six field trials. The flour was then analysed for fAsn concentration; the results are reported in Table S4.1. The fAsn content in the considered samples ranged from 0.99 to 2.82 mmol/kg dry matter (d.m.) for 2018/2019 and from 0.55 to 2.84 mmol/kg d.m. for 2019/2020. Considering the first year of field trials (2018–2019, Figure 4.1), Giambologna, classified as FF, showed the lowest amount of fAsn, reaching 0.99 mmol/kg d.m. in the Foggia field trial, whereas RGT_Montecarlo, classified as FPS, showed the highest amount of fAsn reaching 2.82 mmol/kg d.m. (Table S4.1) in the Voghera field trial. Some cultivars displayed fAsn amounts lower than 1.5 mmol/kg in the three field trials, i.e., Giambologna (classified as FF), Blasco (classified as FPS), Ascott, LG_Absalon, Sothys_CS, SY_Cicerone (all classified as FP), and the two FB genotypes Oregain and Santorin. On the other hand, the two FPS genotypes Lancillotto and RGT_Montecarlo, as well as the FB variety Cosmic, showed fAsn amounts higher than 2 mmol/kg in the three field trials. In the second year of field trials (2019–2020, Figure 4.2), KWS_Coli, a FP variety, showed the lowest amount of fAsn, reaching 0.55 mmol/kg d.m. in the Grosseto field trial, whereas the FPS cultivar SY_Starlord showed the highest amount of fAsn, reaching 2.84 mmol/kg d.m. (Table S4.1) in the Voghera field trial.

Table 4.1. List of the 54 analysed varieties used in the six field trials. The varieties are grouped according to the Italian bread wheat classification, which is based on specific rheologic and analytical parameters [31]. FF: improver wheat; FPS: superior bread making wheat; FP: ordinary bread making wheat; FB: wheat for biscuits.

Italian bread wheat classification	Genotype name	Field trials
FF	Aiace_VST	2019-2020
	Bingo_VST	2019-2020
	Bologna	both years
	Giambologna	2018-2019
	Giorgione	both years
	Izalco_CS	2019-2020
	Metropolis	2018-2019
	Nestore	2018-2019
	Rebelde	both years
FPS	Albagran	2019-2020
	Berta	2019-2020
	Blasco	2018-2019
	Lancillotto	both years
	Pinturicchio	2019-2020
	RGT_Algoritmo	2018-2019
	RGT_Montecarlo	both years
	RGT_Vivendo	2019-2020
	Solindo_CS	2019-2020
	SY_Starlord	2019-2020
	SY_Ural	2019-2020
	Teorema	2018-2019
FP	Amburgo	2018-2019
	Angelico	2018-2019
	Antigua	both years
	Artù_SN	both years
	Ascott	2018-2019
	Ausonio	2018-2019
	Brunelleschi	2018-2019
	Cabiria	2019-2020
	Somtuoso_CS	both years
	Filon	2019-2020
	Ginger	2019-2020
	Guercino	both years
	KWS_Coli	2019-2020
	KWS_Lazulli	2019-2020
	LG_Absalon	2018-2019
	LG_Arnova	both years
	LG_Ascona	both years
	LG_Ayrton	2018-2019

	Poker_VST	2019-2020
	Porticcio	both years
	PR22R58	2018-2019
	Sofolk	2018-2019
	Solehio	both years
	Sothys_CS	both years
	Stromboli	both years
	SY_Capitano	2019-2020
	SY_Cicerone	both years
FB	Avila	both years
	Bramante	both years
	Canaletto	2019-2020
	Cosmic	2018-2019
	Oregrain	2018-2019
	Santorin	2018-2019

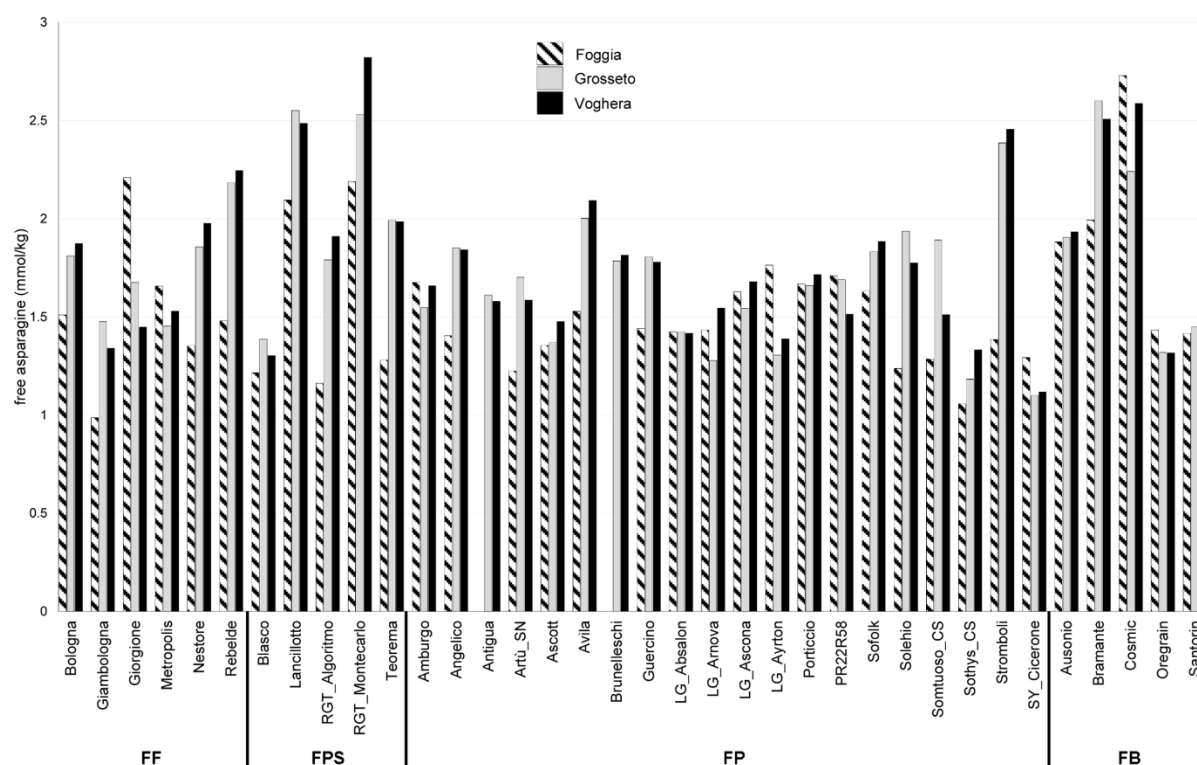


Figure 4.1. Concentration of *fAsn* in the grain of 36 varieties of bread wheat grown in the field trials in 2018–2019 in the three locations Foggia, Grosseto and Voghera. FF: improver wheat; FPS: superior bread making wheat; FP: ordinary bread making wheat; FB: wheat for biscuits.

Some cultivars displayed fAsn amounts lower than 1.5 mmol/kg in the three field trials: the three FF varieties Aiace_VST, Bologna, and Giorgione, the FPS genotype Solindo_CS, and six FP varieties (i.e., Antigua, KWS_Coli, KWS_Lazuli, LG_Arnova, Poker_VST, and SY_Cicerone). No bread wheat variety showed fAsn amounts higher than 2 mmol/kg in the three field trials of the second year. Compared to the first year of field trials, the data of the second year seemed to display a general lower amount of fAsn. In particular, the Grosseto field trial presented very low levels of fAsn, with most of the bread wheat varieties showing a range of fAsn amounts between 0.5 to 1 mmol/kg. Overall, our results were comparable with data obtained in previous works [13,32,33], where fAsn levels ranged between 0.68 and 4.77 mmol/kg d.m. when plants were grown in optimal conditions and treated against pathogens infections. As expected, a variability among the considered genotypes was observed. Although the reported data were preliminary and needed to be confirmed by further evaluations, our analysis suggested that both the environment and genetics may affect fAsn accumulation in the grain of the 54 considered varieties. As reported above, 18 genotypes were sown in both growing seasons during 2018/2019 and 2019/2020. We then focused on the data related to these 18 genotypes to evaluate the possible effect played by the environment and/or by the genotype on fAsn accumulation in the considered bread wheat panel.

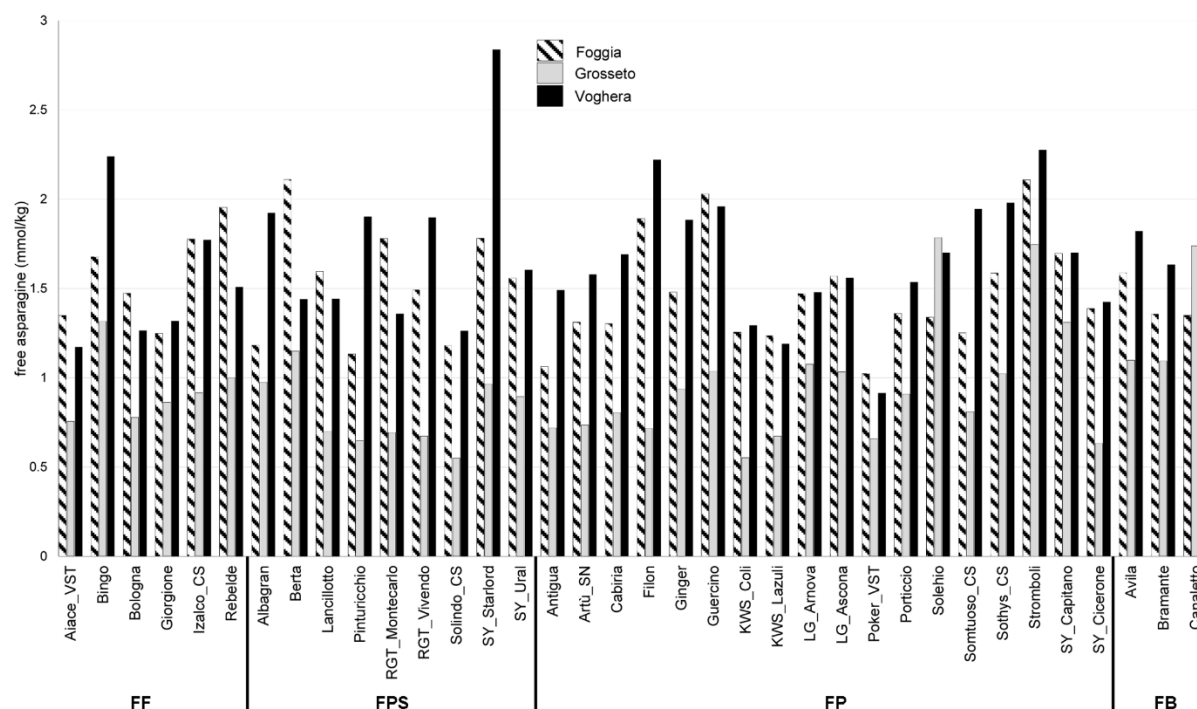


Figure 4.2. Concentration of fAsn in the grain of 36 varieties of bread wheat grown in the field trials in 2019–2020 in the three locations Foggia, Grosseto and Voghera. FF: improver wheat; FPS: superior bread making wheat; FP: ordinary bread making wheat; FB: wheat for biscuits.

4.2.2. Evaluation of the Influence of Growing Environments on Free Asparagine Accumulation

To evaluate a possible influence of the environment, the measurements of fAsn content were compared among the six field trials, considering the data related to each genotype as a whole (Figure 4.3). In the two field trials in Foggia, the median values of fAsn levels in the 18 genotypes were very similar: 1.48 and 1.47 mmol/kg d.m. for the first and the second year, respectively. Similarly, grain harvested in Voghera showed similar median values of fAsn levels in the 18 genotypes between the two field trials: 1.75 and 1.55 mmol/kg d.m. for the first and the second year, respectively. Overall, the Foggia and the Voghera samples showed comparable levels of fAsn. Differently, the genotypes grown in Grosseto in the second year accumulated a lower amount fAsn (median value 0.95 mmol/kg d.m.) compared to those grown in the previous year in the same location (median value 1.81 mmol/kg d.m.). This data is consistent with the previous observation related to the 36 genotypes of the second year

(Figure 4.2), where the Grosseto field trial presented very low levels of fAsn. Interestingly, the samples derived from the field trial in Grosseto in 2018–2019 and 2019–2020 showed the highest and the lowest levels of fAsn content, respectively, compared to those measured in the six field trials considered here. The large effect of crop management and other environmental factors, both alone and interacting with genetic factors (G × E), has a reasonable impact in fAsn accumulation [12]. It has been well demonstrated how differences in growth location and/or growth year may influence fAsn accumulation due to environmental conditions [19,34,35]. Many authors have shown how differences in sulphur or nitrogen availability strongly affect fAsn accumulation [11,13,15,20–24]. In our case, the considered field trials were conducted in the frame of an Italian network of experimental fields (i.e., the Italian “National Network of Common Wheat Variety Comparison”; in Italian “Rete Nazionale di Confronto Varietale del Frumento Tenero”). This network shared similar agricultural practices among field trials, including protocols for fertilization. For this reason, nitrogen and sulphur supply resulted in similar results among the field trials (Table S4.2) and probably did not influence the low fAsn level observed in the 2019–2020 Grosseto field trial. The observed differences in fAsn accumulation in this field trial, when compared to the previous year and to the other locations of Foggia and Voghera, were probably due to environmental factors, such as rainfall and soil water availability, or specific climatic conditions, which highly affected the fAsn accumulation in wheat seeds. This data is in agreement with a previous analysis, which reported that the level of precipitation and temperature during grain development (between heading and harvest) had the greatest effect on fAsn concentration in mature grain [18].

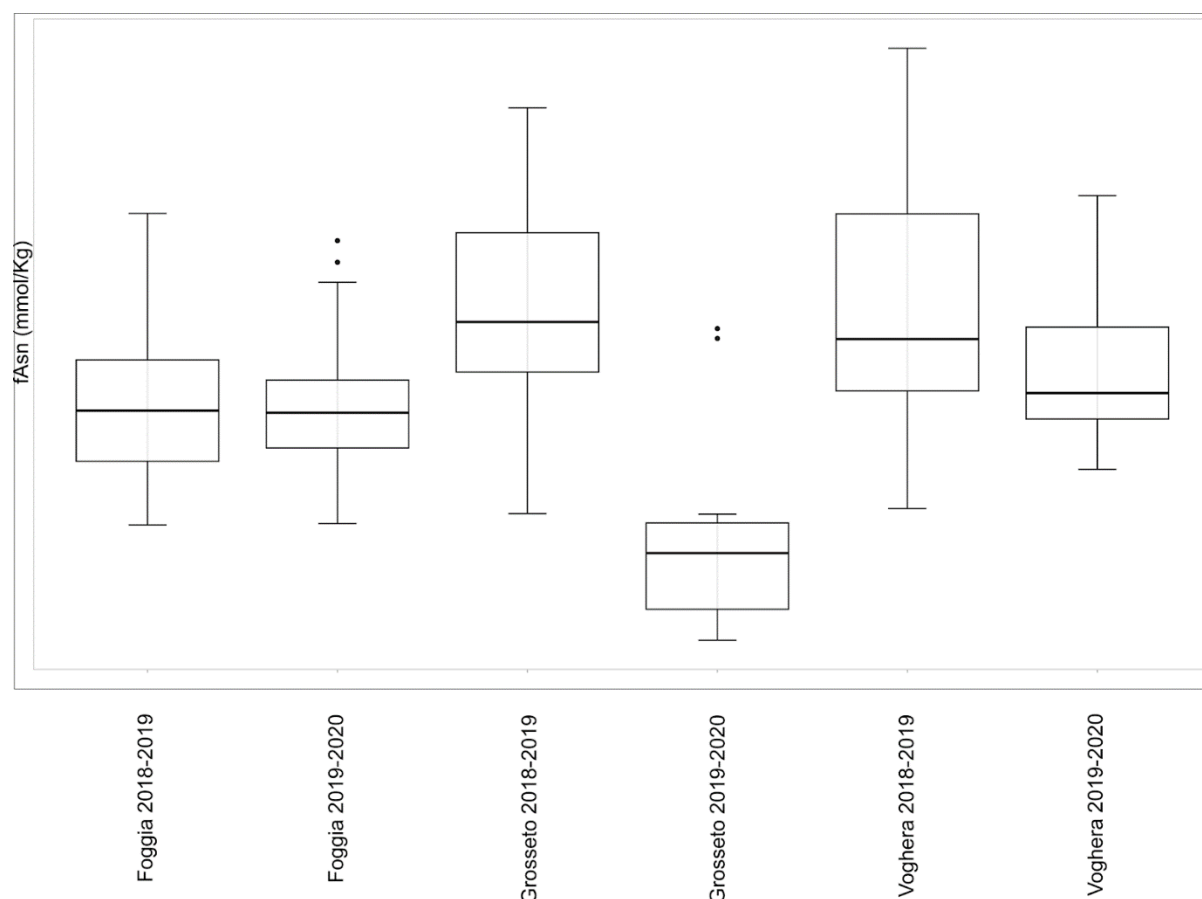


Figure 4.3. Box plots of fAsn measurements in the six considered field trials, considering the data from the 18 genotypes common to the six field trials.

In the two field trials in Foggia, the median values of fAsn levels in the 18 genotypes resulted very similar: 1.48 and 1.47 mmol/kg d.m. for the first and the second year, respectively. Similarly, grain harvested in Voghera showed similar median values of fAsn levels in the 18 genotypes between the two field trials: 1.75 and 1.55 mmol/kg d.m. for the first and the second year, respectively. Overall, Foggia and Voghera samples showed comparable levels of fAsn. Differently, the genotypes grown in Grosseto in the second year accumulated a lower amount fAsn (median value 0.95 mmol/kg d.m.) compared to those grown in the previous year in the same location (median value 1.81 mmol/kg d.m.). This data is consistent with the previous observation related to the 36 genotypes of the second year (Figure 4.2), where Grosseto field trial presented very low levels of fAsn. Interestingly, the samples derived by the field trial in Grosseto in 2018-2019 and 2019-2020 showed the highest and the lowest level of fAsn content, respectively, compared to those measured in the six field trials

here considered. The large effect of crop management and other environmental factors, both alone and interacting with genetic factors ($G \times E$), has a reasonably impact in fAsn accumulation [12]. It is well demonstrated how differences in growth location and/or growth year may influence fAsn accumulation due to environmental conditions [19,34,35]. Many authors showed how differences in sulphur or nitrogen availability strongly affect fAsn accumulation [11,13,15,20–24]. In our case, the considered field trials were conducted in the frame of an Italian network of experimental fields (*i.e.*, the Italian “National Network of Common Wheat Variety Comparison”; in Italian “*Rete Nazionale di Confronto Varietale del Frumento Tenero*”). This network shared similar agricultural practices among field trials, including protocols for fertilization. For this reason, nitrogen and sulphur supply resulted similar among the field trials (Table S4.2) and probably did not weigh on the low fAsn level observed in 2019-2020 Grosseto field trial. The observed differences of fAsn accumulation in this field trial, when compared to the previous year and to the other locations Foggia and Voghera, were probably due to environmental factors, such as rainfall and soil water availability, or specific climatic conditions, which highly affected the fAsn accumulation in wheat seeds. This data is in agreement with a previous analysis, that reported that the level of precipitation and temperature during grain development (between heading and harvest) has the greatest effect on fAsn concentration in mature grain [18].

4.2.3. Evaluation of the influence of genotypic factors on fAsn accumulation

To preliminary characterize a possible effect played by the genotype in fAsn accumulation of the selected bread wheat panel, we compared the fAsn content of the 18 common genotypes considering all the six measurements (three locations for two years; Figure 4.4).

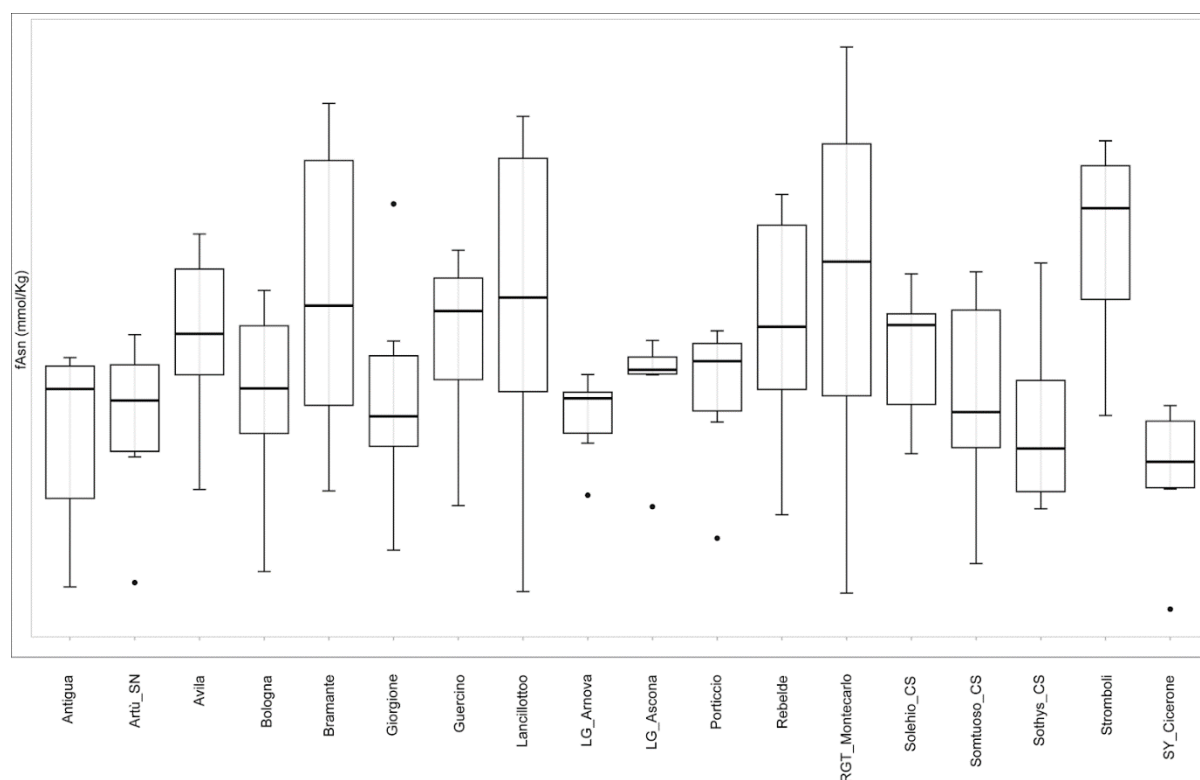


Figure 4.4. Box plots of fAsn measurements from the 18 genotypes present in the six field trials.

The box plots in Figure 4.4 shows a high variability in fAsn content among years and locations for some genotypes. In particular, RGT_Montecarlo and Lancillotto showed the highest variability by accumulating fAsn with differences between maximum and minimum values about 4.06 and 3.64 times, respectively. Other genotypes appeared more stable in terms of fAsn content across year and locations. LG_Arnova and LG_Ascona showed the lowest variability in the amount of fAsn, with differences between maximum and minimum values about 1.44 and 1.63 times, respectively.

Considering the observed levels of fAsn in each genotype in the analysed field trials, SY_Cicerone showed a relatively low and stable amount of fAsn, ranging from 0.63 to 1.42 mmol/kg d.m. (median value 1.21 mmol/kg d.m.). Similarly, the genotype Sothys_CS showed a fAsn content ranging from 1.02 to 1.98 mmol/kg d.m. (median value 1.26 mmol/kg d.m.). If these data will be confirmed in further analyses, these varieties may be utilised to mitigate the acrylamide risk in wheat products and to develop low fAsn accumulator genotypes in future breeding programs. On the other hand, Stromboli showed relatively high fAsn levels, ranging between 1.39 and 2.45 mmol/kg d.m.

(median value 2.19 mmol/kg d.m.). RGT_Montecarlo fAsn values were highly variable between different trials but reached the highest amount of fAsn (2.82 mmol/kg d.m.) of the entire panel and showed a median value (2.07 mmol/Kg d.m.) comparable to Stromboli.

The variability in fAsn accumulation among genotypes is well reported in literature [13,15,17,20,27,33,35]. Despite to the documented effect of environmental factors, the accumulation of fAsn in wheat seeds is a specific trait showing good heritability [15,26,27]. To date, crop management strategies, including ensuring sufficient sulphur in the soil during cultivation [23], and avoiding pathogen infection [21,25], are the most common strategies to reduce fAsn concentrations. Nevertheless, a further crucial point to control fAsn content in wheat seeds is the identification and utilization of wheat genotypes with a strong genetic component in this trait [5]. A recent study highlighted that a natural deletion of a key gene encoding the asparagine synthetase of class 2 of the B genome (*TaASN-B2*) can reduce the amount of fAsn in wheat grain [36]. The presence and absence of this gene was investigated in a panel of common wheat varieties, showing that free asparagine concentrations in field-produced grain were, on average, lower in varieties lacking *TaASN-B2* [36]. In this regard, some breeding programs are already ongoing to reduce the amount of fAsn in wholemeal and refined flour by modulating the expression of key genes, such as *ASN* genes, that are involved in its synthesis inside the kernel.

Previous studies were able to identify specific bread wheat genotypes that were considered low fAsn accumulators and confirmed that selection for low asparagine content is possible [19,21,27,28]. Hence, the analysis of fAsn accumulation in several wheat genotypes sown in different locations may be crucial to identify stable genotypes as well as specific genetic traits associated to fAsn accumulation.

4.3. Materials and Methods

4.3.1. Plant Material and field trials

This study was conducted on 54 bread wheat varieties (*Triticum aestivum* L.) that have been included in Italian “National Network of Common Wheat Variety Comparison” (In Italian: “Rete Nazionale di Confronto Varietale del Frumento Tenero”) coordinated by Council for Agricultural Research and Economics (CREA). The field trials were conducted in three Italian locations (Foggia, Grosseto and Voghera) for two crop years (2018/2019 and 2019/2020). Geographical coordinates, sowing and harvesting date are reported in Table 4.2 for the six field trials.

Table 4.2. Geographical coordinates, sowing and harvesting dates related to all locations and agronomic seasons.

Year	Location	Field coordinates	Sowing date	Harvesting date
2018/2019	Foggia	41°27'45.26"N, 15°30'11.06"E	2018-12-06	2019-06-18
2019/2020	Foggia	41°27'46.03"N, 15°30'10.02"E	2019-12-09	2020-06-11
2018/2019	Grosseto	42°57'14.332"N, 11°5'45.956"E	2019-01-03	2019-07-02
2019/2020	Grosseto	42°57'26.557"N, 11°5'47.68"E	2020-01-05	2020-06-24
2018/2019	Voghera	45°1'44.83"N, 9°1'19.189"E	2018-10-25	2019-07-20
2019/2020	Voghera	45°1'0.3"N, 8°59'44.822"E	2019-12-09	2020-07-12

The applied fertilization inputs are reported in Table S4.2. Weather data was collected during the whole field trials for each location (Table S4.3).

4.3.2 Flour deproteination

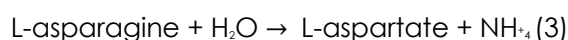
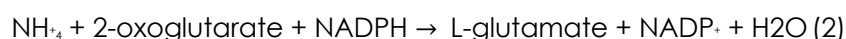
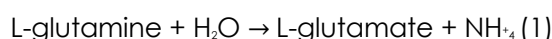
Whole-grain samples were ground through a 0.5 mm grid diameter mesh using a laboratory mill (Cyclotec CT293, FOSS, Hilleroed, Denmark) to obtain a homogeneous powder. Wholemeal flour (5 g) was added to 15 mL of 1 M perchloric acid and shaken for 30 min at room temperature. The suspension was centrifuged at 5300 RCF for 3 min at 4°C to produce a clear extract. Clear supernatant was brought to pH 8 by adding 2 M KOH and adjusted to 50 mL in

a graduated cylinder. The samples were refrigerated in an iced bath for 30 min. Samples were then centrifuged at 5300 RCF for 30 min at 4°C. Clear supernatant was filtered through a 0.45 µm syringe filter and 2 ml was transferred into a 2 mL tube and stored at -20°C for subsequent analysis.

4.3.3 Free asparagine quantification

Quantification of fAsn levels in deproteinated wholemeal flour samples was conducted according to Lecart and co-workers ^[32] with slight modifications, using the K-ASNAM L-Asparagine/L-Glutamine/Ammonia kit (Megazyme, Wicklow, Ireland).

This kit foresees the use of three enzymatic reactions.



Practically, 200 µL of the deproteinated sample were collected and mixed with 100 µL of the pH 4.9 buffer (offered in the K-ASNAM kit) and 10 µL of glutaminase (reaction 1). After a 5 min time lapse at room temperature, 150 µL of the second buffer at pH 8.0 was added together with 100 µL of NADPH and 600 µL of distilled water. After a delay of 10 min at room temperature, 10 µL of glutamate dehydrogenase was added (reaction 2). Subsequently, both the ammonium ions of the sample and those generated by the first reaction react with 2-oxoglutarate and NADPH. The absorbance at 340 nm was then measured to measure the conversion of NADPH to NADP⁺. After measurement, 10 µL of asparaginase was added (reaction 3). In this step, asparagine is hydrolysed to aspartate and ammonium ions by the action of asparaginase. This reaction liberates ammonium ions which enter the second reaction and lead to an additional fall in absorbance at 340nm that is stoichiometric with the concentration of asparagine in the sample. The absorbance was recorded

using a Jasco V-630 spectrophotometer (JASCO, Japan). The concentration of fAsn in the sample was calculated as reported by the manufacturer.

To check the repeatability of the quantification protocol, the same deproteinated whole-grain sample of wheat (cv Palesio) was routinely added to each quantification procedure and measured as control. All the fAsn measurements of this control sample are reported in Table S4.4. The variability among these measurements was estimated about 0.41 mmol/kg and considered as the intrinsic error of the quantification method. The average value of Palesio was 1.48 ± 0.10 mmol/Kg.

4.4. Conclusions

To our knowledge, this work is the first comprehensive analyses for fAsn accumulation in seeds of bread wheat elite varieties relevant for the Italian market. Although these data need to be validated in further field trials, this analysis represents a starting point for the characterization of this genetic material. The food industry requires varieties that can be relied upon to produce grain with low fAsn amounts over a range of environments and harvest years [15,37]. Despite to the environmental role in fAsn accumulation, we identified specific bread wheat varieties showing a good stability among year and location in fAsn concentration. Our data also suggested that the varieties SY_Cicerone and Sothys_CS, with low amount of fAsn in the grain and a low influence from the environment, may be useful for food industry to mitigate the acrylamide risk in wheat derivatives. In contrast, Stromboli and RGT_Montecarlo, that showed high fAsn levels in our analysis, may be useful for study about GxE interaction on this trait. In conclusion, these materials can be useful for further studies to dissect the mechanisms involved in fAsn accumulation in wheat seeds, as well as for studies aimed to reduce the acrylamide-forming potential of wheat varieties.

4.5 Funding

This work was partially funded by the European Regional Development Fund under the ROP of the Lombardy Region ERDF 2014–2020—Axis I “Strengthen technological research, development and innovation”—Action 1.b.1.3 “Support for co-operative R&D activities to develop new sustainable technologies, products and services”—Call hub.

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4.7 Supplementary

Table S4.1: Free asparagine measurements.

	2018-2019							2019-2020					
	Voghera		Grosseto		Foggia			Voghera		Grosseto		Foggia	
	fAsn(mmol/kg)	St.Dev.	fAsn(mmol/kg)	St.Dev.	fAsn(mmol/kg)	St.Dev.		fAsn(mmol/kg)	St.Dev.	fAsn(mmol/kg)	St.Dev.	fAsn(mmol/kg)	St.Dev.
Amburgo	1,66	0,013	1,55	0,011	1,68	0,023	Aiace_VST	1,17	0,009	0,75	0,013	1,35	0,014
Angelico	1,84	0,022	1,85	0,022	1,40	0,008	Albagan	1,92	0,007	0,97	0,002	1,18	0,007
Antigua	1,58	0,007	1,61	0,011	NA	NA	Antigua	1,49	0,003	0,72	0,016	1,06	0,00
Artù_SN	1,58	0,007	1,70	0,006	1,22	0,007	Artù_SN	1,58	0,009	0,74	0,006	1,31	0,007
Ascoff	1,48	0,010	1,37	0,010	1,36	0,006	Avila	1,82	0,009	1,10	0,011	1,59	0,008
Avila	2,09	0,004	2,00	0,004	1,53	0,011	Berta	1,44	0,009	1,15	0,011	2,11	0,009
Ausonia	1,93	0,011	1,90	0,005	1,88	0,009	Bingo	2,24	0,013	1,31	0,013	1,68	0,005
Blasco	1,30	0,012	1,39	0,012	1,22	0,005	Bologna	1,26	0,004	0,78	0,006	1,47	0,009
Bologna	1,87	0,006	1,81	0,013	1,51	0,003	Bramante	1,63	0,002	1,09	0,005	1,36	0,006
Bramante	2,51	0,008	2,60	0,007	1,99	0,004	Cabiria	1,69	0,001	0,81	0,006	1,30	0,003
Brunelleschi	1,81	0,017	1,79	0,011	NA	NA	Canaletto	1,69	0,004	1,74	0,013	1,35	0,01
Cosmic	2,59	0,016	2,24	0,034	2,73	0,023	Filan	2,22	0,010	0,72	0,015	1,89	0,009
Giambologna	1,34	0,017	1,48	0,006	0,99	0,015	Ginger	1,88	0,006	0,94	0,014	1,48	0,005
Giorgione	1,45	0,038	1,68	0,030	2,21	0,041	Giorgione	1,32	0,010	0,86	0,011	1,25	0,009
Guercino	1,78	0,003	1,81	0,002	1,44	0,011	Guercino	1,96	0,015	1,04	0,002	2,03	0,003
Lancillotto	2,48	0,005	2,55	0,005	2,09	0,006	Izalco_CS	1,77	0,008	0,91	0,009	1,78	0,003
LG Absalon	1,42	0,012	1,42	0,001	1,42	0,002	KWS_Coli	1,29	0,005	0,55	0,013	1,25	0,006
LG Arnova	1,54	0,013	1,28	0,031	1,43	0,008	KWS_Lazuli	1,19	0,010	0,67	0,011	1,24	0,012
LG Ascona	1,68	0,010	1,54	0,005	1,63	0,019	Lancillotto	1,44	0,007	0,70	0,009	1,59	0,014
LG Ayrton	1,39	0,025	1,30	0,008	1,76	0,008	LG Arnova	1,48	0,005	1,08	0,007	1,47	0,006
Metropolis	1,53	0,008	1,45	0,011	1,66	0,012	LG Ascona	1,56	0,004	1,03	0,014	1,57	0,007
Nestore	1,98	0,015	1,86	0,012	1,35	0,016	Pinuricchio	1,90	0,012	0,65	0,008	1,13	0,009
Oregrain	1,32	0,007	1,32	0,008	1,43	0,015	Poker_VST	0,91	0,003	0,66	0,004	1,02	0,003
Porticcio	1,72	0,005	1,66	0,006	1,67	0,009	Porticcio	1,53	0,011	0,91	0,009	1,36	0,002
PR22R58	1,51	0,007	1,69	0,007	1,71	0,010	Rebelle	1,51	0,010	1,00	0,009	1,96	0,004
Rebelle	2,25	0,028	2,18	0,008	1,48	0,020	RGT_Montecarlo	1,36	0,003	0,69	0,007	1,78	0,002
RGT_Algoritmo	1,91	0,022	1,79	0,010	1,16	0,022	RGT_Vivendo	1,90	0,015	0,67	0,033	1,49	0,024
RGT_Montecarlo	2,82	0,011	2,53	0,012	2,19	0,029	Solehio	1,70	0,033	1,78	0,007	1,34	0,012
SANTORIN	1,41	0,004	1,45	0,008	1,42	0,003	Solindo_CS	1,26	0,012	0,55	0,008	1,18	0,007
Sofolk	1,88	0,003	1,83	0,008	1,63	0,004	Somtuoso_CS	1,95	0,003	0,81	0,002	1,25	0,002
Solehio	1,77	0,005	1,94	0,005	1,24	0,002	Solhys_CS	1,98	0,003	1,02	0,005	1,59	0,007
Somtuoso_CS	1,51	0,008	1,89	0,011	1,29	0,001	Stromboli	2,28	0,005	1,75	0,010	2,11	0,018
Solhys_CS	1,33	0,017	1,18	0,016	1,06	0,014	SY_Capitano	1,70	0,015	1,31	0,017	1,70	0,012
Stromboli	2,45	0,008	2,39	0,015	1,39	0,009	SY_Cicerone	1,42	0,007	0,63	0,010	1,39	0,012
SY_Cicerone	1,12	0,013	1,10	0,016	1,29	0,008	SY_Starlord	2,84	0,004	0,96	0,002	1,78	0,008
Teorema	1,98	0,013	1,99	0,006	1,28	0,015	SY_Ural	1,60	0,002	0,89	0,002	1,56	0,011

Table S4.2: Fertilizer inputs.

Location/year	Treatment date	Fertilizer	Quantity
Voghera 2018/2019	2019-02-18	Amm. nitrate 33,5	200kg/ha
	2019-04-02	"	200kg/ha
	2019-05-01	"	100kg/ha
Voghera 2019/2020	2020-03-24	Amm. nitrate 33,5	200kg/ha
	2020-04-24	Urea 46	100kg/ha
Grosseto 2018/2019	2019-01-09	NP 18/46	100kg/ha
	2019-03-12	Urea 46	180kg/ha
	2019-04-18	Urea 46	150kg/ha
Grosseto 2019/2020	2020-03-10	NP 18/46	150kg/ha
	2020-03-16	Urea 46	100kg/ha
	2020-04-15	Urea 46	
Foggia 2018/2019	post sowing	N	81kg/ha
Foggia 2019/2020	2019-11-26	NP 18/46	200kg/ha
	2020-04-02	SULFAN 27	200kg/ha

Fertilizer	Fertilizer components	Components %	Additional informations
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Amm. nitrate 33,5	Nitrate N Ammonium N	16.90% 16.60%	
NP 18/46	NH ₄ -N P ₂ O ₅ P ₂ O ₅	18,0 % 46,0 % 43,0 %	soluble in neutral ammonium citrate and in water water soluble
Urea 46	NH ₂	46,0 %	
Sulfan 27 N + 12 So3	Nitrate N Ammonium N So3	13.10% 13.9% 12%	

Table S4.3: Weather data.

Grosseto 2018/2019							
year	month	Decade	Rain (mm)	T max (°C)	T min (°C)	T ave (°C)	
2018	OCT	I	n.a.	n.a.	n.a.	n.a.	
2018	OCT	II	n.a.	n.a.	n.a.	n.a.	
2018	OCT	III	n.a.	n.a.	n.a.	n.a.	
2018	NOV	I	n.a.	n.a.	n.a.	n.a.	
2018	NOV	II	n.a.	n.a.	n.a.	n.a.	
2018	NOV	III	n.a.	n.a.	n.a.	n.a.	
2018	DEC	I	n.a.	n.a.	n.a.	n.a.	
2018	DEC	II	n.a.	n.a.	n.a.	n.a.	
2018	DEC	III	n.a.	n.a.	n.a.	n.a.	
2019	JAN	I	0.00	10.97	-2.28	3.68	
2019	JAN	II	1.55	12.30	-1.06	4.70	
2019	JAN	III	5.45	8.59	-0.41	4.21	
2019	FEB	I	6.78	13.59	3.00	8.00	
2019	FEB	II	0.11	16.00	-0.80	6.84	
2019	FEB	III	0.00	16.85	0.54	7.89	
2019	MAR	I	0.00	17.92	2.48	9.67	
2019	MAR	II	0.62	15.84	2.95	9.07	
2019	MAR	III	0.12	19.69	3.65	11.37	
2019	APR	I	4.11	17.79	3.42	10.37	
2019	APR	II	3.04	19.86	4.87	12.05	
2019	APR	III	3.87	20.55	7.61	13.81	
2019	MAY	I	1.39	19.61	5.71	12.65	
2019	MAY	II	5.82	19.36	8.55	13.58	
2019	MAY	III	6.24	22.61	11.09	16.31	
2019	JUN	I	0.00	29.22	10.49	20.09	
2019	JUN	II	0.00	31.68	12.17	22.34	
2019	JUN	III	0.00	35.46	15.65	26.16	

Foggia 2018/2019							
year	month	Decade	Rain (mm)	T max (°C)	T min (°C)	T ave (°C)	
2018	OCT	I	17.30	23.12	14.27	18.69	
2018	OCT	II	0.30	23.09	13.91	18.50	
2018	OCT	III	83.60	21.60	12.20	16.90	
2018	NOV	I	10.30	19.52	10.76	15.14	
2018	NOV	II	33.30	16.75	7.53	12.14	
2018	NOV	III	35.00	14.11	7.74	10.93	
2018	DEC	I	15.40	15.55	6.03	10.79	
2018	DEC	II	21.00	9.87	3.24	6.56	
2018	DEC	III	2.70	13.50	4.03	8.77	
2019	JAN	I	45.40	10.72	3.79	7.25	
2019	JAN	II	3.50	9.60	0.45	5.02	
2019	JAN	III	12.00	13.72	4.28	9.00	
2019	FEB	I	13.70	16.28	6.60	11.44	
2019	FEB	II	20.00	11.40	1.05	6.22	
2019	FEB	III	0.40	17.06	3.41	10.24	
2019	MAR	I	42.20	14.14	3.37	8.75	
2019	MAR	II	28.80	12.09	5.15	8.62	
2019	MAR	III	1.20	15.08	5.11	10.09	
2019	APR	I	3.60	17.09	4.98	11.03	
2019	APR	II	25.20	14.65	5.50	10.07	
2019	APR	III	7.40	20.51	8.22	14.36	
2019	MAY	I	1.20	23.98	11.72	17.85	
2019	MAY	II	7.00	23.33	12.92	18.13	
2019	MAY	III	1.60	26.21	13.23	19.72	
2019	JUN	I	2.30	30.48	14.36	22.42	
2019	JUN	II	0.00	34.41	17.34	25.87	
2019	JUN	III	0.00	32.88	19.09	25.98	

Voghera 2018/2019							
year	month	Decade	Rain (mm)	T max (°C)	T min (°C)	T ave (°C)	
2018	OCT	I	1.60	15.72	15.40	15.57	
2018	OCT	II	11.20	15.82	15.60	15.69	
2018	OCT	III	62.80	14.14	13.80	13.98	
2018	NOV	I	36.40	13.17	13.00	13.10	
2018	NOV	II	4.00	9.56	9.40	9.48	
2018	NOV	III	19.20	6.13	5.94	6.03	
2018	DEC	I	0.20	5.73	5.41	5.57	
2018	DEC	II	12.20	1.00	0.81	0.91	
2018	DEC	III	0.80	3.15	2.88	3.01	
2019	JAN	I	0.20	1.92	1.51	1.71	
2019	JAN	II	6.40	3.40	3.02	3.21	
2019	JAN	III	9.40	-0.83	-1.10	-0.98	
2019	FEB	I	42.20	2.59	5.84	2.46	
2019	FEB	II	0.00	6.32	7.65	6.08	
2019	FEB	III	0.00	8.11	9.92	7.88	
2019	MAR	I	0.00	10.39	9.88	10.15	
2019	MAR	II	10.00	10.34	10.80	10.11	
2019	MAR	III	0.20	11.25	11.50	11.00	
2019	APR	I	17.00	11.86	12.60	11.67	
2019	APR	II	13.00	12.94	13.80	12.76	
2019	APR	III	24.40	14.16	13.20	13.97	
2019	MAY	I	40.40	13.67	13.60	13.45	
2019	MAY	II	24.00	13.67	17.30	13.73	
2019	MAY	III	40.80	17.65	21.60	17.46	
2019	JUN	I	0.60	22.10	23.00	21.85	
2019	JUN	II	0.00	23.47	27.10	23.23	
2019	JUN	III	0.00	27.53	26.60	27.29	

Grosseto 2019/2020							
year	month	Decade	Rain (mm)	T max (°C)	T min (°C)	T ave (°C)	
2019	OCT	I	1.79	23.86	10.28	16.67	
2019	OCT	II	1.04	23.51	10.64	16.17	
2019	OCT	III	4.97	23.72	12.14	16.91	
2019	NOV	I	9.22	17.85	8.71	12.82	
2019	NOV	II	15.51	11.69	6.59	9.20	
2019	NOV	III	4.45	12.92	6.85	9.53	
2019	DEC	I	1.83	11.15	3.95	7.37	
2019	DEC	II	3.01	11.06	4.66	7.61	
2019	DEC	III	16.50	12.78	1.55	6.26	
2020	JAN	I	0.00	13.40	-0.66	5.15	
2020	JAN	II	2.00	11.94	1.00	5.91	
2020	JAN	III	4.65	14.63	4.19	8.79	
2020	FEB	I	0.00	15.42	3.21	8.85	
2020	FEB	II	0.24	17.02	2.74	9.52	
2020	FEB	III	0.22	17.40	4.01	10.44	
2020	MAR	I	5.00	15.01	5.18	10.21	
2020	MAR	II	0.00	18.58	7.08	12.32	
2020	MAR	III	1.43	15.26	4.71	9.71	
2020	APR	I	0.00	21.41	4.39	12.77	
2020	APR	II	2.62	21.35	8.13	14.25	
2020	APR	III	3.11	21.22	9.32	14.95	
2020	MAY	I	0.00	23.22	10.83	17.09	
2020	MAY	II	3.02	23.53	14.41	18.94	
2020	MAY	III	1.22	26.24	12.43	19.67	
2020	JUN	I	3.24	25.86	13.49	19.66	
2020	JUN	II	2.24	27.08	13.38	20.00	
2020	JUN	III	0.05	32.13	16.40	24.58	

Foggia 2019/2020							
year	month	Decade	Rain (mm)	T max (°C)	T min (°C)	T ave (°C)	
2019	OCT	I	17.80	23.66	12.13	17.89	
2019	OCT	II	0.00	25.66	11.77	18.71	
2019	OCT	III	0.60	24.75	10.51	17.63	
2019	NOV	I	27.30	20.28	11.52	15.90	
2019	NOV	II	40.70	18.10	9.41	13.75	
2019	NOV	III	24.80	17.11	8.85	12.98	
2019	DEC	I	15.10	14.34	7.63	10.98	
2019	DEC	II	10.20	14.87	4.59	9.73	
2019	DEC	III	4.40	13.28	5.17	9.22	
2020	JAN	I	45.40	10.72	3.79	7.25	
2020	JAN	II	3.50	9.60	0.45	5.02	
2020	JAN	III	12.00	13.72	4.28	9.00	
2020	FEB	I	13.70	16.28	6.60	11.44	
2020	FEB	II	20.00	11.40	1.05	6.22	
2020	FEB	III	0.40	17.06	3.41	10.24	
2020	MAR	I	42.20	14.14	3.37	8.75	
2020	MAR	II	28.80	12.09	5.15	8.62	
2020	MAR	III	1.20	15.08	5.11	10.09	
2020	APR	I	3.60	17.09	4.98	11.03	
2020	APR	II	25.20	14.65	5.50	10.07	
2020	APR	III	7.40	20.51	8.22	14.36	
2020	MAY	I	7.10	24.26	9.68	16.97	
2020	MAY	II	5.02	28.01	14.76	21.39	
2020	MAY	III	27.40	23.98	11.90	17.94	
2020	JUN	I	2.50	26.85	14.65	20.75	
2020	JUN	II	9.30	28.28	15.08	21.68	
2020	JUN	III	2.50	31.80	16.68	24.24	

Voghera 2019/2020							
year	month	Decade	Rain (mm)	T max (°C)	T min (°C)	T ave (°C)	
2019	OCT	I	7.40	15.70	14.60	15.52	
2019	OCT	II	79.20	14.82	15.20	14.71	
2019	OCT	III	131.40	15.37	9.05	15.29	
2019	NOV	I	40.60	9.24	6.20	9.15	
2019	NOV	II	100.80	6.35	8.68	6.27	
2019	NOV	III	74.80	8.85	4.76	8.76	
2019	DEC	I	19.60	5.00	4.89	4.88	
2019	DEC	II	33.80	5.04	4.55	4.97	
2019	DEC	III	38.60	4.88	1.81	4.71	
2020	JAN	I	0.20	2.09	2.94	1.95	
2020	JAN	II	41.80	3.18	2.81	3.06	
2020	JAN	III	0.00	3.13	5.45	2.97	
2020	FEB	I	3.40	5.75	7.59	5.60	
2020	FEB	II	10.00	7.99	7.79	7.78	
2020	FEB	III	1.60	8.18	6.62	7.98	
2020	MAR	I	49.00	6.94	10.50	6.78	
2020	MAR	II	0.80	10.90	7.87	10.72	
2020	MAR	III	0.60	8.19	10.90	8.02	
2020	APR	I	n.a.	11.37	14.40	11.13	
2020	APR	II	n.a.	14.84	15.50	14.61	
2020	APR	III	n.a.	15.86	17.70	15.66	
2020	MAY	I	n.a.	18.17	17.60	17.93	
2020	MAY	II	n.a.	17.93	19.60	17.78	
2020	MAY	III	n.a.	19.87	18.80	19.78	
2020	JUN	I	n.a.	19.15	19.70	18.96	
2020	JUN	II	n.a.	20.10	24.50	19.89	
2020	JUN	III	n.a.	24.99		24.74	

Table S4.4: Free asparagine measurements of the standard genotype.

Samples	Asn		mean	st.dev.
	mmol/kg	St.Dev.	mmol/kg	mmol/kg
Palesio rep. 1	1.55	0.010	1.51	0.10
Palesio rep. 2	1.32	0.008		
Palesio rep. 3	1.44	0.013		
Palesio rep. 4	1.46	0.018		
Palesio rep. 5	1.66	0.017		
Palesio rep. 6	1.63	0.022		
Palesio rep. 7	1.56	0.001		
Palesio rep. 8	1.68	0.019		
Palesio rep. 9	1.46	0.002		
Palesio rep. 10	1.49	0.010		
Palesio rep. 11	1.42	0.012		
Palesio rep. 12	1.51	0.011		
Palesio rep. 13	1.44	0.012		
Palesio rep. 14	1.64	0.010		
Palesio rep. 15	1.54	0.001		
Palesio rep. 16	1.71	0.009		
Palesio rep. 17	1.73	0.010		
Palesio rep. 18	1.36	0.004		
Palesio rep. 19	1.53	0.003		
Palesio rep. 20	1.42	0.003		
Palesio rep. 21	1.48	0.015		
Palesio rep. 22	1.42	0.010		
Palesio rep. 23	1.40	0.006		
Palesio rep. 24	1.51	0.003		
Palesio rep. 25	1.46	0.023		
Palesio rep. 26	1.52	0.003		
Palesio rep. 27	1.49	0.007		
Palesio rep. 28	1.46	0.011		
Palesio rep. 29	1.46	0.012		

Chapter 5. Mitigation of acrylamide formation in wood oven baked pizza base using wheat grain with low free asparagine content

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Journal of the Science of Food and Agriculture (submitted)

The results presented in this chapter are presented in the manuscript:

Covino C., Tafuri A., Sorrentino A., Masci S., Baldoni E., Sestili F., Villalonga R., Masi P. Mitigation of acrylamide formation in wood oven baked pizza base using wheat grain with low free asparagine content. Submitted to *Journal of the Science of Food and Agriculture*

As part of my thesis work, I directly participated in the selection of genotypes (5.2.1 Raw material, chemical and enzymatic kits) that have been used in this work. In addition, I performed the quantification of free asparagine (5.2.4 *Free Asparagine quantification*) and analysed the data related to the production of acrylamide (paragraph 5.3.6. *Acrylamide levels in wood oven baked pizza base*)

Abstract

Background: In wheat-derived bakery products, the quantity of free Asparagine (fAsn) has been identified as a key factor in acrylamide (AA) formation. Based on this assumption, four varieties of common wheat (*Triticum aestivum*, L.), *Stromboli*, *Montecarlo*, *Sothys* and *Cosmic*, selected for their different fAsn content inside the grain, were studied to evaluate their potential in the production of pizza with reduced AA levels. To this purpose, wholemeal and refined flours were obtained from each variety.

Results: The fAsn content ranged from 3.30 to 0.25 mmol/kg, with higher values for wholemeal flours which also showed greater amount of ash, fibre and damaged starch than refined wheat flours. All types of flours were separately used to produce wood oven baked pizza base, according to the "TSG" EU Regulation (97/2010). Acrylamide reduction in the range 47-68 % was found for all the selected wheat cultivars, compared with a commercial flour, with significantly lower values registered when refined flour was used. Moreover, refined leavened dough samples showed decreased levels of fAsn and reducing sugars due to the fermentation activity of yeasts.

Conclusion: This study clearly shows that a reduced asparagine content in wheat flour is a key factor in the mitigation of AA formation in pizza base. Furthermore, it was confirmed that pizza made with wholemeal flours exhibit lower levels of RDS (rapidly digestible starch) and RAG (rapidly available glucose) compared to that prepared with the refined flour.

Keywords: acrylamide reduction, asparagine, wheat flour, starch digestibility.

5.1 Introduction

Neapolitan pizza represents Italian food all around the world but, being made by wheat flour and cooked at very high temperature, it can be an acrylamide (AA) source, an organic compound formed in high starchy heat-treated foods. AA has negative effects on health (and is classified as a “probable human carcinogen”).^{1,2} In addition, many studies have shown that AA can be genotoxic, neurotoxic, and toxic to the reproductive system.^{3,4,5} The main route to AA production in heated treated foods is the Maillard Reaction, which occurs between carbonylic compounds and amino compounds like free amino acids, especially free asparagine (fAsn) at high temperature.⁶ Food and Drug Organization/World Health Organization and the European Food Safety Authority analysed products on the markets and published guidelines to minimize AA risks.^{7,8} Previous studies have used strategies to reduce AA formation in foods, such as minimizing reducing sugars and asparagine content, increasing moisture, heating at a lower temperature, and shortening the processing time.^{9,10} Reference levels of AA in baked foods are 50-800 µg/kg, while 750-4000 µg/kg is accepted for French fries and coffee (EU Reg. 2017/2158),¹¹ but pizza baked in a wood oven is not mentioned. Although AA levels in most baked foods are tolerable, targeted interventions to reduce this toxic substance are always welcome. Wheat flour is a strongly starchy matrix and contains fAsn in a wide range, 7.4-66.4 mg/100g (corresponding to a range of 0.56 to 5.03 mmol/kg).¹²⁻¹⁴ In the EU Regulation (2017/2158),¹¹ one of the strategies for reducing AA formation is the elimination of asparagine, using the enzyme L-Asparaginase,^{15,16} but this is a rather expensive procedure. Selection of wheat genotypes with low asparagine content in the grain, including the use of natural and induced mutations,¹⁷ could be another efficient strategy to reduce AA levels in bakery products made by wheat flour, like pizza.

Free asparagine accumulation in bread wheat grain (*Triticum aestivum* L.) may consistently vary among wheat cultivars and is strongly influenced by environmental factors and cultivation management.¹⁸⁻²² Nevertheless, this trait

shows good heritability (Rapp et al., 2018). Thus, the identification of cultivars accumulating low and stable fAsn levels across different growing environments may help to mitigate the AA risk in wheat products (Oddy et al., 2022; Raffan and Halford., 2019; Tafuri et al., submitted).^{17,21,22}

Hence, asparagine is the crucial AA precursor in bakery products.^{13,23} Nevertheless, AA formation depends also on other several factors, such as water absorption, fibre and damaged starch in flour.²⁴ Springer et al.²⁵ demonstrated that baked foods made from less intensively milled flours had lower levels of AA. Additionally, AA content has been shown to increase as the damaged starch content of wholemeal flour increases.²⁶ For wholemeal wheat flours with a higher fibre content, an increase in dough development time and dough water absorption was observed, affecting the formation of AA in the end products.²⁷ Another important factor that can influence AA formation is dough fermentation.²⁴ The content of asparagine and reducing sugars, the main precursors of AA formation, in leavened dough may depend on the time and the temperature of fermentation and the amount of yeast (*S. cerevisiae*) added in the dough.²⁸ Many studies also demonstrated that the consumption of asparagine during dough fermentation can be an effective way to mitigate the formation of AA in baked products.^{10,23,28}

The objective of this study was to explore the possibility of using flours from selected bread wheat varieties with low fAsn content in the grain for the preparation of wood oven baked pizza base with reduced AA levels. To this purpose, four bread wheat varieties were selected for the high or low fAsn content in the grain based on previous analysis.²² The wholemeal and refined flours of these genotypes were characterised for their proximal composition and hydration indexes and for fAsn, reducing sugars and damaged starch contents. Both wholemeal and refined flours were then used in the production of pizza dough samples, which were analysed for their structure as well as for fAsn and reducing sugars. Most importantly, AA content and *in vitro* starch digestibility in wood oven pizza base were assessed to analyse the differences between wholemeal and refined flours in terms of the potential for both AA

reduction and glucose release, whose absorption in the human small intestine is associated with the glycaemic response.

5.2 Materials and methods

5.2.1 Raw material, chemical and enzymatic kits

Four elite varieties of bread wheat (*Triticum aestivum* L.) were selected based on the characterisation of the different levels of free asparagine.²² The cultivar *Stromboli* was provided by ISTA Sementi (Lodi, Italy); *RGT_Montecarlo* (hereinafter referred to as *Montecarlo*) by RAGT Italia, Masi Torello, (FE, Italy), *Sothys_CS* (hereinafter referred to as *Sothys*) by Semetruria (Tarquinia, VT, Italy), and *Cosmic* by Nova Sem (Genola, CN, Italy). Hexane, methanol, water for HPLC and all other reagents used were of analytical grade and purchased from CARLO ERBA Reagents S.r.l. Total Starch Assay Kit (AA/AMG), Digestible and Resistant Starch Assay Kit, K-ASNAM L-Asparagine/L-Glutamine/Ammonia and Starch Damage Assay (K-SDAM) kits were purchased from Megazyme (Wicklow, Ireland) and used according to manufacturer's instructions.

5.2.2 Wheat grain milling

Stromboli, *Montecarlo*, *Sothys* and *Cosmic* wheat grains were milled using a hammer mill (Perten Instruments, Sweden). The wholemeal flours were obtained by using a 0.50 mm grid. For the refined flours, the grains were first ground in the same mill with a 2.00 mm grid and then, the milled flours were sieved in the vibrating screen to remove the bran and obtain refined flours with grain size < 0.20 mm.

5.2.3 Characterisation of wheat flours

Moisture and ash content were determined by gravimetric method according to procedures outlined by AOAC.²⁹ Five g of sample were placed in crucible and incubated at 105°C for 24 h to calculate the moisture content. Subsequently, the samples were moved to a muffle furnace at 550°C for 6 h to calculate the total ash. The total nitrogen and protein content of wheat flours,

wholemeal (W) and refined (R), were determined by the Kjeldhal method.³⁰ Samples were subjected to mineralization in Speed Digester K-425 connected to a Scrubber K-415 (Büchi, Switzerland), then subsequently distilled with a Distiller K-350 (Büchi, Switzerland). Total starch (%) in wheat flours was determined by using Total Starch Assay Kit (AA/AMG) (Megazyme, Wicklow, Ireland). Fats in samples were analysed through soxhlet extraction method using Büchi Extraction System B-811 (Büchi Labortechnik AG, Flawil, Switzerland). Total fibre was calculated as the difference of carbohydrates, proteins, and fats.

5.2.4 Free Asparagine quantification

Wholemeal and refined flour (5 g) was added to 15 mL of 1 M perchloric acid and shaken for 30 min at room temperature. The suspension was centrifuged at 5300×g for 3 min at 4 °C to produce a clear extract. Clear supernatant was brought to pH 8 by adding 2 M KOH and adjusted to 50 mL in a graduated cylinder. The samples were refrigerated in an iced bath for 30 min. Samples were then centrifuged at 5300×g for 30 min at 4°C. Clear supernatant was filtered through a 0.45 µm syringe filter and 2 mL was transferred into a 2 mL tube and stored at -20°C for subsequent analysis. Quantification of free asparagine levels in deproteinated wholemeal and refined flour samples was conducted according to Lecart et al.³¹ with slight modifications, using the K-ASNAM L-Asparagine/L-Glutamine/Ammonia kit (Megazyme, Wicklow, Ireland). The absorbance was recorded using a Jasco V-630 spectrophotometer (JASCO, Japan). The concentration of asparagine in the sample was calculated as reported by the manufacturer.

5.2.5 Damaged starch and reducing sugars determination

Damaged starch was evaluated in wholemeal and refined flours for the four wheat cultivars by using the Starch Damage Assay (K-SDAM) kit and calculated as g/100 g of flour, according to manufacturer's instructions.

The 2,4-dinitrosalicylic acid (DNS) assay for reducing sugars was used according to the method of Miller³² (1959). Samples preparation for the extraction of soluble substances in the different doughs is described in detail in Covino et al.³³ Twenty μL of sample extract were reacted with DNS reagent, and reducing sugars were determined by reading absorbance at 540 nm, through UV/VIS spectrophotometer (V-730, Jasco, Sud Africa). Samples were analysed in triplicate ($n=3$) and data were reported as mean $\pm$ SD (Standard Deviation).

5.2.6 Determination of WAI, WSI and SP

The water uptake index (WAI) and solubility index (WSI) of wheat flour samples were determined using the method of Anderson et al.³⁴ with a slight modification. Wheat flour samples (1 g) were weighted into a 50 mL centrifuge tube containing with 15 mL deionized water. All centrifuge tubes were transferred into the Orbital Shaker (Forma Scientific) at 25 °C for 20 min at 185 rpm. Samples were centrifuged at 5000 rpm for 30 min. Pellet of samples was weighted and supernatant was placed in the stove at about 105°C overnight. WAI and WSI index and swelling power (SP) of wheat flour samples were calculated according to the following formulas:

$$WAI = \frac{\text{wet pellet weight (g)}}{\text{dry sample weight (g)}} \quad (1)$$

$$WSI = \frac{\text{soluble solids weight (g)}}{\text{dry sample weight (g)}} \times 100 \quad (2)$$

$$SP = \frac{\text{wet pellet weight (g)}}{\text{dry sample weight (g)} \times (1 - \frac{WSI}{100})} \quad (3)$$

5.2.7 Dough and pizza preparation

In the mixing cell of a Farinograph-AT (Brabender, Duisburg, Germany), wholemeal and refined flours (300 g) were kneaded together with 61 % water, 2.8 % salt, 0.17 % dry yeast (on the flour weight basis) at 26 °C and 63 min⁻¹ of

pasting speed. Mixing of ingredients (~ 3-4 min), for the preparation of pizza dough samples, lasted until the dough development time on the farinogram was reached, but stopping the test before reaching the degree of softening (UB). The leavening was carried out at 22 °C and 80 % RH for 8 h. After leavening, some samples were frozen and then freeze-dried and ground (< 0.50 mm) for subsequent analysis. In parallel, other pizza dough samples were rolled out until a circular-shaped disc was formed and baked in a wood oven at 485 ± 30 °C, monitored with a thermal imaging camera, for 60 seconds.¹⁶ Afterwards, the pizza base samples were allowed to cool and then cut into pieces, frozen, freeze-dried, and eventually ground (< 0.50 mm) for subsequent analysis.

5.2.8 Volume image analysis

Volume of dough balls (~250 g) over leavening time was calculated using an image analysis protocol. Leavening kinetic for each sample was monitored over 8 h by digital image acquisition, analysing the leavening at 30 min intervals. The height (h) and width (l) of the dough balls were calculated through Image J software, and the volume (V) during selected leavening times was determined according to the method described in Covino et al.³³ Calculated volume at each interval of leavening was expressed as the V_t/V_0 index, where V_t is the volume measured at time t and V_0 is the initial volume of the pizza dough, and plotted as a function of leavening time.

5.2.9 SEM analysis of dough

SEM analyses were conducted for the evaluation of dough samples made with wholemeal (W) and refined (R) flours. Lyophilized dough slices were placed on specimen holders and coated with gold by means of DC sputtering (Sputter and Carbon Coater QUORUM Q150RS) in order to make the sample conductive. The microstructure of samples was observed at a magnification of 1500× with SEM microscope (Jeol, JSM-6335F, Tokyo, Japan) with a 20 kV

acceleration voltage. Representative micrographs from all the samples were selected.

5.2.10 Acrylamide determination

Freeze-dried and ground wood oven baked pizza base samples were subjected to AA extraction and determination following the procedures previously described in Covino et al.¹⁶ The recovery of standard AA was in the range of 86-96%.

5.2.11 *In vitro* digestible starch

Digestible and resistant starch in the pizza base samples obtained with both wholemeal and refined flours of each considered variety were determined by AOAC method 2017.16 by using the Digestible and Resistant Starch Assay Kit (Megazyme, Wicklow, Ireland) with minor modification reported in Covino et al.³³ Rapidly Digestible Starch (RDS) and Slowly Digestible Starch (SDS) were the percentage of starch hydrolysed by amylolytic enzymes within 20 and 120 min. Resistant Starch (RS) accounts for the amount of starch not digested even after 240 min of enzymatic reaction. The percentage of digestible and resistant starch in the samples was calculated as reported by the manufacturer.

Each measurement was carried out in triplicate. Starch Digestion Rate Index (SDRI), an indicator of *in vitro* starch digestibility, was calculated as RDS divided by Total Starch (TS=RDS+SDS+RS) for all samples. Rapidly Available Glucose (RAG) was determined as a predictor of the potential glycaemic response derived from the ingestion of these food items.³⁵

5.2.12 Statistical test

All experiments and analytical measurements were run in triplicate (n=3) and the data were expressed as mean $\pm$ SD. Means of each parameter were analysed by analysis of variance (ANOVA) using different test, Bonferroni and Post Hoc Tukey. Differences between samples at 5% level ($p < 0.05$) were considered as significant. Principal Component Analysis (PCA) was carried out

for correlating samples with different wheat varieties (*Stromboli*, *Montecarlo*, *Sothys*, *Cosmic*) prepared with wholemeal (W) and refined (R) flours, using the calculated variables, AA, fAsn, damaged starch, reducing sugars, and fibre. Statistical analyses were performed using XLSTAT software (version 2022).

5.3 Results and discussion

5.3.1 Wheat flour composition

Wholemeal (W) and refined (R) flours, obtained from the grains of the bread wheat varieties *Stromboli*, *Montecarlo*, *Sothys*, and *Cosmic*, were physico-chemical characterized. The obtained results about total starch, protein, fat, moisture, ash and fibre are shown in Table 5.1. The two types of flours presented specific differences in physico-chemical characteristics. Considering the carbohydrate content, expressed as total starch (%), *Stromboli* and *Montecarlo* showed similar values for wholemeal (W) and refined flours (R) (Table 5.1). Differently, *Sothys* and *Cosmic* presented higher values in the refined flours, equal to 72.30 ± 2.23 % and 73.52 ± 2.45 %, respectively, than in wholemeal flour, with total starch content of 62.23 ± 2.78 % and 64.41 ± 1.94 %, respectively (Table 5.1).

Overall, wholemeal flours presented a higher content of ash, protein, fat and fibre than refined flours (Table 5.1). In wheat flours, fats ranged from 1.5 to 2.5 %.³⁶ The samples here analysed showed a very low fat content, ranging between 0.89 ± 0.13 % and 1.39 ± 0.12 %.

Considering the protein content, *Stromboli* and *Montecarlo* showed higher values than *Sothys* and *Cosmic*, both in refined and wholemeal flours. Specifically, *Stromboli* flours showed the highest protein content (about 14 %), followed by *Montecarlo* with approximately 13 %, *Cosmic* flours with around 10 %, and finally *Sothys* with the lowest content of around 9 %. About one-sixth of the protein content is constituted by the soluble protein fractions (albumins and globulins), and the remainder by gliadins and glutenins which, during the mixing stage of the ingredients in the presence of water, form the gluten, responsible of the visco-elastic properties of the dough.³⁷ Therefore, flour with

12% protein contains about 10% gluten.³⁶ Generally, there is an inverse relationship between the amount of protein and starch, and this is in accordance with the obtained results. In fact, both *Cosmic* and *Sothys* refined flours showed the highest values of starch and the lowest values of protein content, compared to the other flours. Protein content is one of main parameter that mostly influences flour strength (W).³⁸ In general, the flour is defined as weak flour when it contains 8-9% protein and $W < 170$, medium flour with protein values of 10-12% and $W 180 \div 260$, and strong flours with 13-14% protein content and $W 280 \div 350$.³⁸ A strong flour has a robust and elastic structure that prevents the dough breakdown, making it more suitable than a weak flour in long leavening applications, such as bread, pizza, or *panettone*. Differences in total protein content can be attributed to varietal difference, geographic location of cultivation and fertilization.²⁷

It is well documented that wholemeal flours also have more fibre than refined ones. Indeed, during flour processing, such as milling and refining, the bran layers are selectively removed, and refined wheat flour typically consists of a limited amount of dietary fibre (2-3%). Dietary fibre is the third abundant component of wheat flour after starch and protein. It has been well established that the fibre contributes to the prevention of several diet-related diseases, *i.e.*, heart disease, colon cancer, diabetes and obesity.²⁷ In the reported analysis, among wholemeal flours, *Sothys* showed the highest fibre content (15.46 ± 0.25 %), whereas among refined flour *Cosmic* presented the lowest fibre value equal to 3.97 ± 0.06 % (Table 5.1). The fibre content may particularly affect the dough development. Indeed, an increase in dough development time and dough water absorption was observed for wholemeal wheat flours with a higher fibre content.³⁹ On the other hand, a decreased specific volume of the dough and a possibly undesirable texture was observed for flours with high fibre content.³⁹

A further important parameter, which can be considered as an indicator of flour shelf-life, is the moisture. In the flours examined for this study, moisture values ranged from a minimum of 8.14 ± 0.04 % to a maximum of 10.94 ± 0.08 %

(Table 5.1), within the acceptable limit of no more than 10% for long-term storage of wheat flour.⁴⁰

Finally, considering the ash content, that is an indication of the mineral content of a flour or a food,²⁷ as expected, the refined flours showed lower values compared to wholemeal flours, caused by the higher degree of refining that results from more intensive milling.⁴¹

Table 5.1 Proximal composition of wholemeal (W) and refined (R) flours from selected bread wheat varieties.

Type of flour	Variety	Moisture	Starch	Fibre ^o	Protein	Fat	Ash
				(%)			
W	<i>Stromboli</i>	8.14±0.04 ^d	64.34±0.31 ^a	10.68±0.29 ^c	14.14±0.37 ^a	1.23±0.15 ^{ab}	1.49±0.02 ^b
	<i>Montecarlo</i>	9.76±0.04 ^c	64.52±1.65 ^a	10.60±0.13 ^c	12.53±0.12 ^b	1.02±0.03 ^b	1.58±0.04 ^a
	<i>Sothys</i>	10.19±0.06 ^b	62.23±2.78 ^a	15.46±0.25 ^a	9.27±0.06 ^d	1.39±0.12 ^a	1.48±0.01 ^b
	<i>Cosmic</i>	10.61±0.04 ^a	64.41±1.94 ^a	12.35±0.03 ^b	10.08±0.04 ^c	1.36±0.10 ^{ab}	1.20±0.01 ^c
R	<i>Stromboli</i>	9.41±0.14 ^b	65.96±1.69 ^a	7.90±0.55 ^{a*}	14.25±0.36 ^a	1.17±0.01 ^{ab}	1.32±0.04 ^{a*}
	<i>Montecarlo</i>	9.26±0.04 ^b	66.41±1.97 ^a	8.68±0.09 ^{a*}	13.34±0.01 ^{b*}	0.89±0.13 ^b	1.42±0.01 ^{a*}
	<i>Sothys</i>	10.89±0.15 ^a	72.30±2.23 ^{b*}	6.14±0.32 ^{b*}	8.70±0.08 ^{c*}	0.90±0.04 ^{b*}	1.08±0.01 ^{b*}
	<i>Cosmic</i>	10.94±0.08 ^a	73.52±2.45 ^{b*}	3.97±0.06 ^{c*}	9.42±0.09 ^{c*}	1.32±0.09 ^a	0.84±0.03 ^{c*}

^o Calculated as complement to 100%. Significance calculated by ANOVA statistical test with Bonferroni (p<0.05). Different lower-case letters indicate significant differences between selected wheat cultivars. Significant differences between wholemeal (W) and refined (R) flours are marked by *.

5.3.2 Hydration indexes of wholemeal and refined flours

The functional properties of flours were evaluated at 25°C by measuring the water absorption index (WAI), water solubility index (WSI) and swelling power (SP) of wholemeal and refined flours (Table 5.2). WAI and WSI can be used to quantify water content, soluble solids and the ability of particles to absorb water relative to dry matter.³⁴ Water absorption index (WAI) is a measure of the volume occupied by the starch granule after swelling in an excess of water, while solubility index (WSI) account for the amount of soluble substances released from the starch granule; finally, welling power (SP) is the ratio of weight of the wet sediment to the initial weight of dry starch.⁴²

These parameters were affected by the flour type in some genotypes. Wholemeal flours showed a WAI in the range of 2.01±0.12 ÷ 2.24±0.02, higher

than refined flours with a range of $1.97 \pm 0.01 \div 2.02 \pm 0.02$. This result may be due to the higher fibre content in these samples, which increases the water retention during hydration. In a previous study,⁴³ wheat flour, with a moisture content of 12.57 ± 0.28 %, showed WAI values of 1.92 ± 0.06 , WSI values of 2.09 ± 0.26 % and SP values of 1.96 ± 0.07 . WSI values obtained for the analysed flours, both wholemeal and refined, were higher than those reported by Rodriguez-Sandoval et al.⁴³ This difference is probably due by a different level of milling, since the flour used by Rodriguez-Sandoval et al.⁴³ was refined type 00, whereas the flour used in the present study was less refined, as shown by the high fibre values (Table 5.1), and by a higher content of water-soluble substances, such as sugars, amino acids, mineral salts, etc. Indeed, WSI values of wholemeal wheat flours were found to be higher in a range of 6.25 ± 0.22 % $\div$ 4.56 ± 0.16 % than refined flours, where calculated values were 5.64 ± 0.09 % $\div$ 3.25 ± 0.02 %. Instead, the presented results agree to the study reported by Ma et al.⁴⁴, where WAI and WSI for wheat flour were found to be 1.58 ± 0.11 and 4.89 ± 0.12 %, respectively. Notably, *Cosmic* refined flour has the lowest fibre content (3.98%) and the lowest solubility index (3.25 ± 0.02 %), indicating that total soluble substances were lower. The functional properties (WSI, WAI, SP) of flours are influenced by their morphology, processing, and composition. For example, wheat flour fats are present at lower levels but could significantly affect swelling and water absorption. In addition, the technological quality of protein is also related to the water absorption of flour.⁴⁵ The indexes are also affected by the amylose/amylopectin ratio and the characteristics of amylose and amylopectin in terms of molecular weight/distribution, degree and length of branching, and conformation.⁴³ Further, Stauffer affirmed that damaged starch granules absorb about four times as much water as intact granules and increase dough water absorption.³⁶

Table 5.2: Hydration indexes (WAI, WSI, SP) of wholemeal and refined flours.

Flour	Cultivar	WAI	WSI (%)	SP
W	<i>Stromboli</i>	2.01±0.12 ^a	4.56±0.16 ^b	2.11±0.12 ^a
	<i>Montecarlo</i>	2.07±0.05 ^a	6.25±0.22 ^a	2.21±0.06 ^a
	<i>Sothys</i>	2.24±0.02 ^b	5.78±0.09 ^{ab}	2.38±0.02 ^b
	<i>Cosmic</i>	2.15±0.03 ^a	5.19±0.72 ^{ab}	2.27±0.04 ^a
R	<i>Stromboli</i>	2.01±0.03 ^a	3.88±0.49 ^b	2.09±0.04 ^a
	<i>Montecarlo</i>	2.02±0.02 ^a	5.64±0.09 ^{a*}	2.14±0.01 ^a
	<i>Sothys</i>	1.97±0.01 ^{a*}	3.87±0.08 ^{b*}	2.05±0.02 ^{a*}
	<i>Cosmic</i>	1.97±0.09 ^{a*}	3.25±0.02 ^b	2.04±0.01 ^{a*}

Significance calculated by ANOVA statistical test with Bonferroni ($p < 0.05$). Different lower case letters indicate significant differences between selected wheat cultivars. Significant differences between wholemeal (W) and refined (R) flours are marked by *.

5.3.3 Volumetric index in different wheat dough

The leavening performance was monitored during 8 h by capturing digital images, from which the volumetric index of leavened pizza dough samples was obtained through analysing the different samples at 30 min time intervals. Generally, during leavening, the dough volume increases, due to the fermentative activity of yeasts, which convert the fermentable sugars into mainly CO₂ and ethanol. Dough expansion is a result of the diffusion of CO₂ in the air bubbles formed in the matrix during fermentation.⁴⁶ The curve trend describing the volume expansion of dough prepared with wholemeal and refined wheat flours is very similar to the yeast growth curve and, therefore, can be considered as a sequence of three distinct phases.⁴⁷ During the first phase, called latency phase, the dough does not undergo considerable volume changes, as the yeasts have just begun their fermentation activity and the CO₂ produced takes time to diffuse into the air bubbles of the dough matrix. In the next stage, exponential phase, the dough expands exponentially reaching the maximum volume and finally, its value remains constant. In the presented analyses, the dough volumetric index (V_t/V_0), obtained with wholemeal flours (Figure 5.1A), showed the typical sigmoidal trend; in fact, after a latency phase in the first hours of leavening, there was an exponential

increase in volumetric index to then reach constant values in the final time. In these samples, the exponential increase phase was shorter than that observed in the doughs obtained with refined flours (Figure 5.1B), in which the leavening was more intense, in terms of an increase in volume index that exceeds the doubling of volume index in the final hours. Wholemeal flours usually have a higher fibre content due to the presence of bran, compared to dough samples prepared with refined flours. Particularly, it has been shown that the insoluble fibre in bran can interact with gluten proteins, disrupting the continuity of the gluten network.⁴⁸ Thus, the interaction between fibre and gluten proteins inhibits the expansion of dough volume and, as a result, doughs made from wholemeal flours would need less time to reach maximum volume.⁴⁹ In fact, whole wheat bakery product is often associated with many distinctive attributes such as low dough volume, firm and gritty texture, dark and rough crust and crumb appearance, bitter flavour, and reduced shelf-life.⁵⁰

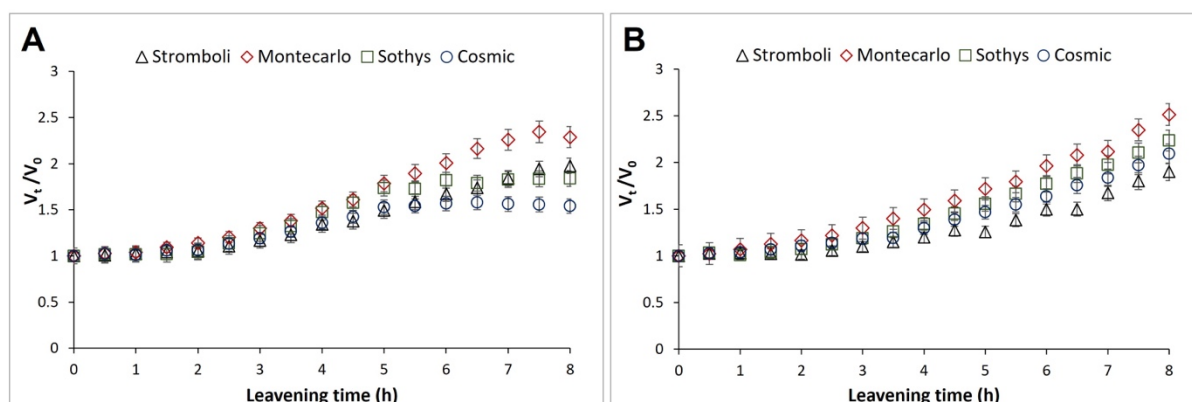


Figure 5.1: Volumetric index during leavening of pizza dough prepared with wholemeal (A) and refined (B) flours from selected wheat cultivars.

5.3.4 Asparagine content in flour and dough

The fAsn is a major precursor of acrylamide formation in baked foods, especially in wheat derivatives.^{51,52} For this reason, fAsn levels were determined in wholemeal and refined flours of the four selected varieties and in the respective doughs after 8 h of leavening (Figure 5.2). Considering wholemeal flour, *Stromboli* and *Montecarlo* showed the highest fAsn content with

3.30±0.29 mmol/kg and 1.603±0.15 mmol/kg, respectively. On the contrary, *Sothys* and *Cosmic* showed the lowest levels of fAsn, with 1.242±0.24 mmol/kg and 1.019±0.309 mmol/kg, respectively. The differences in fAsn level observed among genotypes were consistent with literature data, since it is well known that this trait is highly influenced by both environmental and genetic factors.^{19,21,53} Specifically, data related to *Stromboli*, *Montecarlo* and *Sothys* were in agreement with previous observation: *Stromboli* and *Montecarlo* showed “high fAsn levels” in all the considered field trials, whereas *Sothys* resulted a “fAsn low accumulator” genotype. Conversely, *Cosmic* presented a high amount of fAsn in previous analysis.²² This different behaviour is probably due to a minor stability in fAsn accumulation of this wheat genotype across different growing environments. Indeed, it is well documented that some wheat genotypes may be more influenced by environmental factors for this trait.^{19,54}

Overall, whole grain flours had a higher fAsn content than the corresponding refined flours for all the considered varieties (Figure 5.2). Therefore, it can be argued that asparagine content in wheat grains also increases with protein content.⁵⁵ The obtained results present a good correlation between asparagine and protein content (Table 5.1 and Figure 5.2). In the study by Corol et al.¹⁸, asparagine levels were determined in 150 genotypes, ranging from 2.42-11.81 mmol/kg of wholemeal flours. Fredriksson et al.²³ analysed different types of wheat grains and found that wholemeal flours had an average fAsn content of 3.78 mmol/kg, while refined flours had lower values, which were around 1.51÷2.27 mmol/kg. These data are comparable to our data related to *Stromboli*, that presented 3.30±0.29 mmol/kg and 1.852±0.197 mmol/kg of fAsn in wholemeal and refined flour, respectively. This trend is observable also for the other varieties. In particular, *Sothys* and *Cosmic* refined flours showed the lowest asparagine content, at 0.430±0.068 mmol/kg and 0.255±0.077 mmol/kg, respectively.

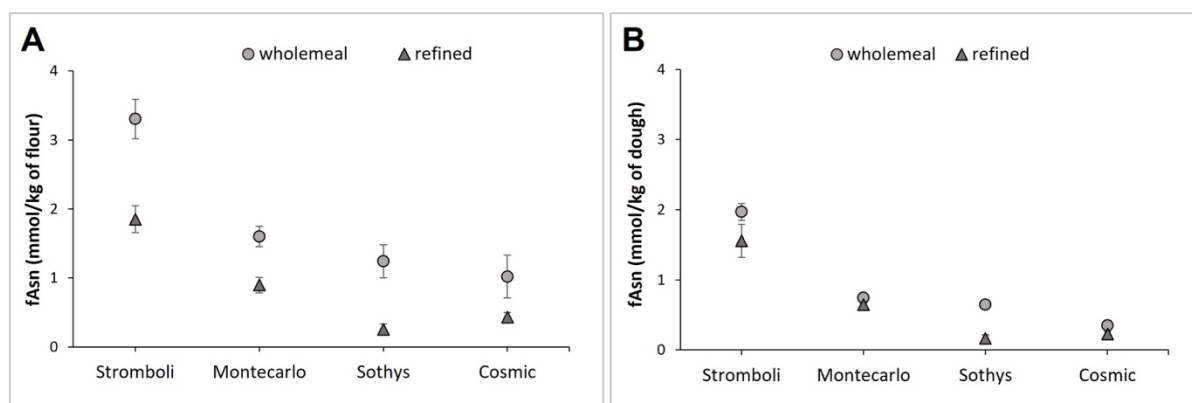


Figure 5.2: fAsn content (mmol/kg) in wholemeal and refined flours from selected wheat cultivars (A), and in corresponding leavened pizza dough (B).

In all the analysed wheat cultivars, 8 h of leavening caused a decrease in asparagine content in both wholemeal and refined flours (Figure 5.2). In wholemeal, fAsn content in leavened dough decreased from 40.4 % in *Stromboli* to 65.8 % in *Cosmic*. In refined flour, the reduction was lower: from 16.1 % in *Stromboli* to 65.8 % in *Cosmic*. This data suggested the breakdown of asparagine by proteolytic enzymes during dough leavening and it is consistent with previous observations (Claus et al., 2006; Fredriksson et al., 2004; Mustafa et al., 2009; Katsaiti and Granby, 2016).^{13,23,55,56} Fredriksson et al.²³ observed a higher fAsn decrease, around 80 %, in wholemeal flour after 6 h of leavening. Similarly, Katsaiti and Granby⁵⁷ registered a depletion of fAsn content in the dough from 68 to 89 %. These differences are probably due to different factors, such as fermentation time and yeast type, that significantly influenced this process, as reported by the authors. The results reported in this study confirmed that an optimization of leavening can be a further strategy to decrease fAsn content in wheat derivatives, and consequently the AA amount in baked products.

5.3.5 Gluten composition, damaged starch and reducing sugars

The four varieties chosen in this study showed the same gliadin composition as the bread wheat cultivar Pricama, used as standard by CREA-DC (Tavazzano (LO), Italy), for the technical examinations needed for listing in the National Register of new plant varieties. The high molecular weight glutenin subunits

(HMW-GS) composition is reported in Table 5.3 (kindly communicated by Dr C. Delogu, CREA-DC, Tavazzano (LO), Italy).

Table 5.3: High Molecular Weight glutenin subunits (HMW-GS) compositions and hardness characteristics of the selected wheat cultivars.

Cultivar	Glu-A1	Glu-B1	Glu-D1	Texture
<i>Stromboli</i>	1	6+8	5+10	Medium
<i>Montecarlo</i>	Nulli	7+8	2+12	Medium
<i>Sothys</i>	Nulli	7+8	5+10	Medium Hard
<i>Cosmic</i>	Nulli	7+9	3+12	Soft

Texture (measured as grain hardness) is a very important parameter influencing milling, baking and final use of wheat flour. Grain hardness includes three main classes: soft and hard hexaploids (bread wheats, *Triticum aestivum* L.), and very hard durum (durum wheats, *Triticum turgidum* L. ssp. *durum* (Desf.)). Bread wheat varieties can show intermediate hardness. *Cosmic* is a bread wheat variety with soft texture, used mostly for cookies and biscuits, thanks also to its HMW-GS composition at the *Glu-D1* locus. Soft wheat flours typically have a low starch damage, and this was confirmed by our analyses (Figure 5.3A). The other three varieties are used for breadmaking and have a medium (*Stromboli* and *Montecarlo*) or medium-hard (*Sothys*) texture (Table 5.3). Surprisingly, *Sothys* showed the lower amount of starch damage (Figure 3A), and the second lower amount of reduced sugar, both as refined or wholemeal flour (Figure 5.3B), whereas *Stromboli* and *Montecarlo* had the highest levels of both parameters.

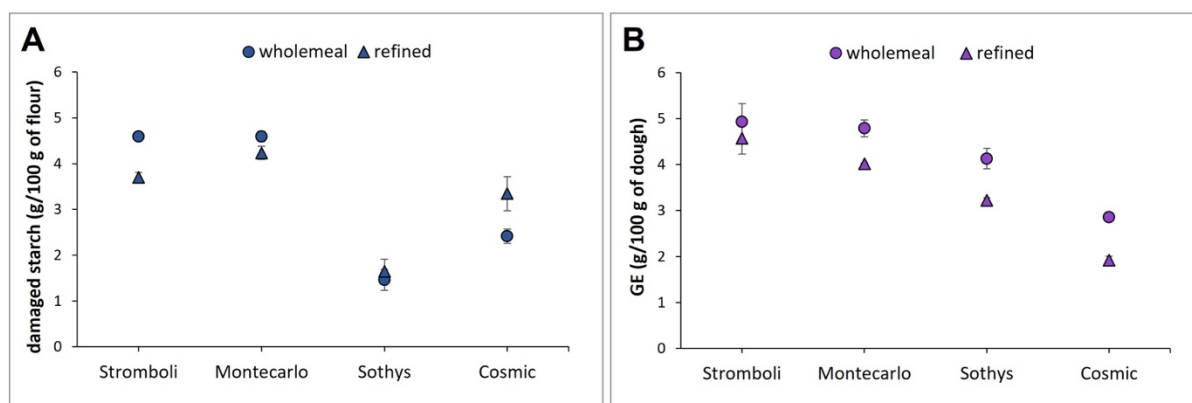


Figure 5.3: Damaged starch in wholemeal and refined flours from selected wheat varieties (A), and reducing sugar content in corresponding pizza dough after leavening (B). GE, glucose equivalent reducing sugars.

The content of reducing sugars, analysed in the doughs after 8 h of leavening, was found to be higher for samples made from wholemeal than refined flours, except for *Stromboli*, which did not show significant differences, and whose values were the highest for both types of doughs (Figure 5.3B). For the other genotypes, the reducing sugar values passed from 4.785 ± 0.397 to 4.019 ± 0.343 (*Montecarlo*), 4.127 ± 0.222 to 3.220 ± 0.057 (*Sothys*) and 2.858 ± 0.018 to 1.922 ± 0.084 (*Cosmic*) g GE/100 g of dough with significant differences between wholemeal and refined samples. It is reported that different flours, used for dough preparation, show different values of reducing sugars also depending on genotype and damaged starch.¹³ Generally, flours have a reducing sugar content ranging between $0.39 \div 0.93$ g/100 g for wheat, as studied by Claus et al.¹³, and $1.503 \div 2.027$ g/100 g, as reported by Hamlet et al.⁵⁸ In the dough, the presence of yeast (*S. cerevisiae*) can substantially change the content of reducing sugars and asparagine, which are the main precursors of acrylamide formation in baked foods.²⁴ The significant higher reducing sugars content in the dough after leavening, for *Stromboli* and *Montecarlo* genotypes, indicated a higher net production than consumption of reducing sugars, resulting from the degradation of starch granules by yeast, which could be attributed to the higher content of damaged starch in the initial flour.²⁴ *Cosmic* refined flour had more damaged starch than the

wholemeal counterpart, making available more available reducing sugars for the fermentation activity of yeast, because the damaged starch is much more susceptible to the action of α -amylase than intact starch.³⁶ This resulted in an improving of leavening kinetic, as shown in Figure 5.1, associated with a lower amount of final reducing (Figure 5.3). Surprisingly, *Sothys*, while showing the lowest levels of damaged starch in both wholemeal and refined flours, compared to all other flours, exhibited high values of reducing sugars after the leavening processing (Figure 5.3). Since the kinetics of leavening curve for *Sothys* dough was similar to that of *Cosmic*, it can be assumed that the reducing sugars production rate, by the amylolytic activity of endogenous enzymes in the flour and/or from yeast, should be higher than the yeast metabolic consumption. This could justify the higher residual amount of reducing sugar in the doughs.

5.3.6 Acrylamide levels in wood oven baked pizza base

The acrylamide content in the pizza base samples is shown in Figure 5.4. Considering the wholemeal flour samples, the highest AA values were found in pizza base prepared with *Stromboli* and *Montecarlo* flours, that reached 1075.78 ± 62.53 and 1176.45 ± 28.12 $\mu\text{g}/\text{kg}_{\text{dw}}$ (dry weight) of pizza base, respectively, while *Sothys* and *Cosmic* showed comparable lower AA content (801.42 ± 129.29 and 945.00 ± 77.78 $\mu\text{g}/\text{kg}_{\text{dw}}$). Although refined flours seemed to follow an overall trend of reduction in AA formation, compared to the corresponding wholemeal flours, the differences were not significant, except for *Montecarlo* flours (Figure 5.4). In fact, in this sample, a reduction of about 40 % was observed between refined and wholemeal pizza base.

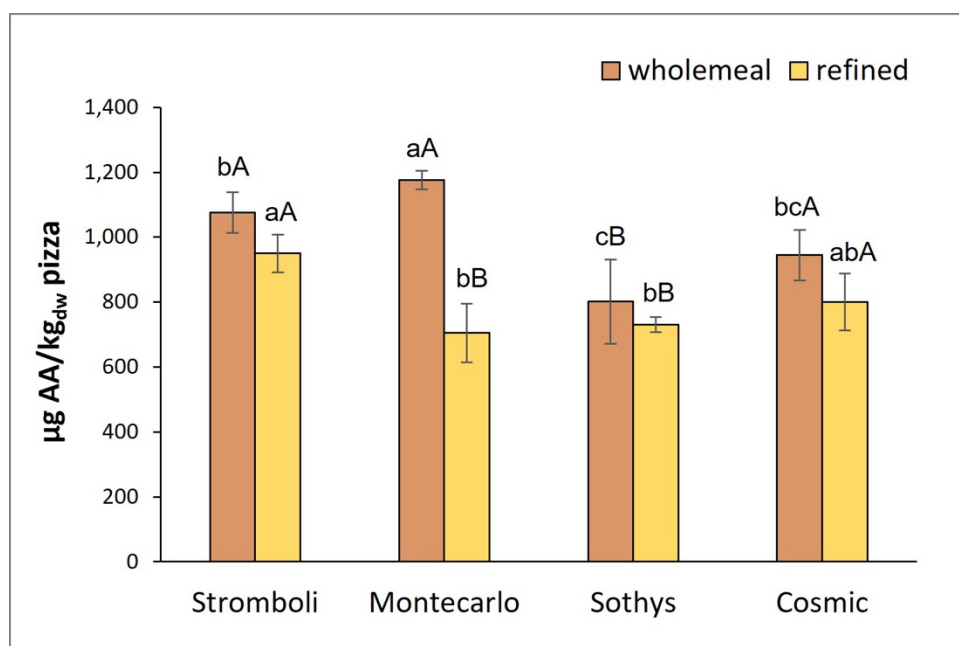


Figure 5.4: AA content ($\mu\text{g}/\text{kg}_{\text{dw}}$) in wood oven baked pizza samples made from wholemeal and refined flours from selected wheat cultivars. Letters indicate samples significance calculated by ANOVA statistical test with Post Hoc Tukey ($p < 0.05$). Lower case letters indicate differences between samples from different cultivar. Capital letters refer to differences between wholemeal and refined flours.

In a previous study, AA content for the pizza base prepared with a refined commercial “00” flour, using the same recipe, leavening and baking conditions of the present study, was found to be $2210 \pm 205 \mu\text{g}/\text{kg}_{\text{dw}}$.¹⁶ Compared to this value, certainly flours obtained from the selected wheat cultivars showed a better impact on AA formation. Specifically, a reduction in AA content was found in the range of 47-68 % with respect to the control, with higher reduction values for refined flours.

5.3.7 Correlation Analysis through PCA test

Chemometric analysis of the dataset was performed through Principal Component Analysis (PCA). Analysed variables, such as fibre, damaged starch, reducing sugars, fAsn in dough, and AA content of wholemeal (W) and refined (R) samples were compared. Figure 5.5 shows the standardised loading plot of the variables corresponding to the first two eigenvectors, which concentrate 81.24 % of the total variance (F1: 56.54 % and F2: 24.70 %). The

Principal Component F1 is positively associated with AA, fAsn and reducing sugars. As expected, AA was positively correlated with its precursors, asparagine and reducing sugars. The Principal Component F2 is positively associated with fibre and damaged starch, but with a low contribution of the other variables.

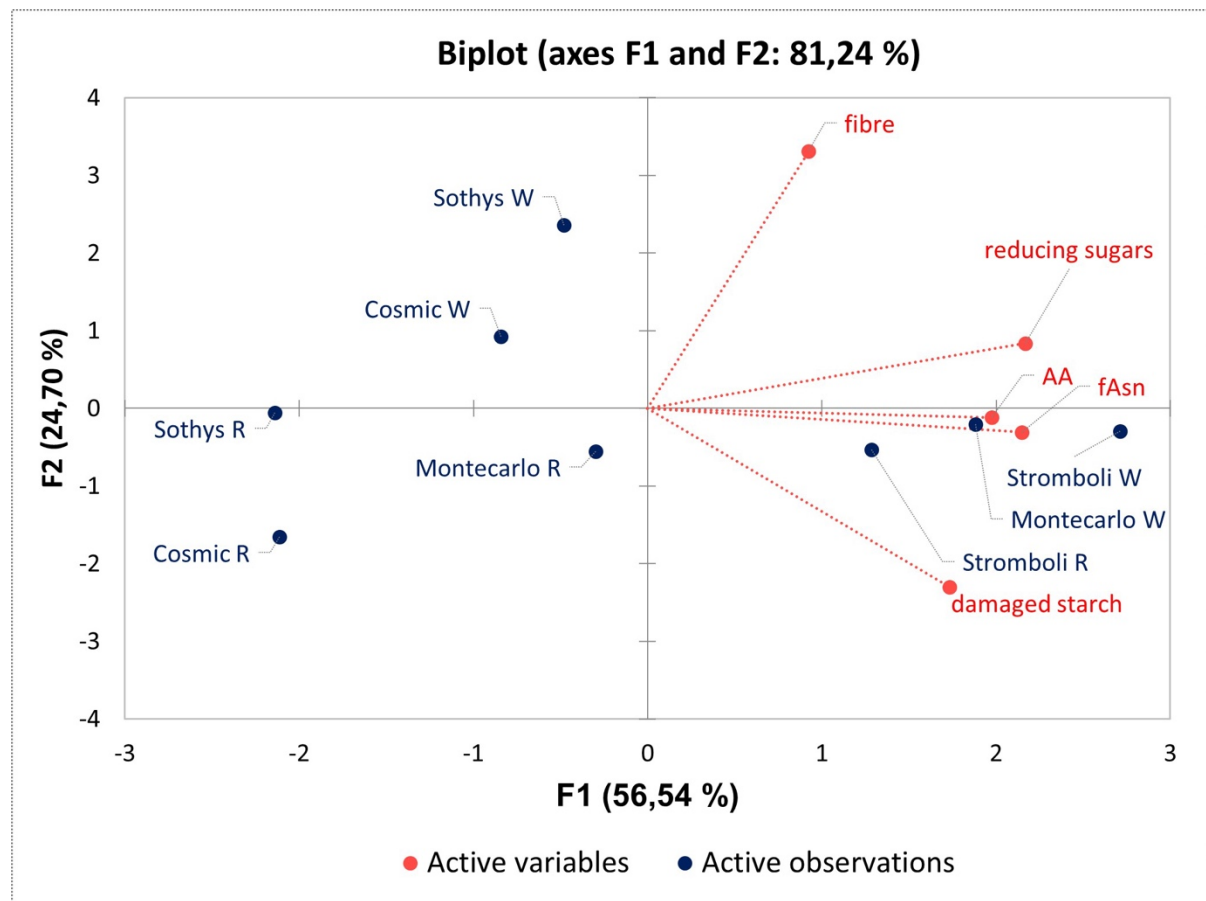


Figure 5.5: Principal component analysis of different wheat cultivar samples. W, wholemeal and R, refined flours.

Stromboli R, *Montecarlo W* and *Stromboli W* samples were characterised by the analysed variables, especially AA, fAsn and reducing sugars were more present in the mentioned samples. This result is in agreement with results shown in Figures 5.2B, 5.3B and 5.4.

Furthermore, the analysed variables were less present in the samples located in the third and fourth quadrants (Figure 5.5), in particular the damage starch.

For *Montecarlo* cultivar, refining of flour positively affected acrylamide formation; indeed, *Montecarlo* R sample was located in the opposite quadrant, compared to its integral counterpart. The first component (F1) explained more the behaviour of *Sothys* R and *Cosmic* R, which were negatively characterised by the variables AA, fAsn and reducing sugars. In contrast, *Sothys* W and *Cosmic* W had the opposite behaviour compared to the variables associated with F1 and seemed to be more explained by the fibre variable. This analysis allowed us to better interpret the experimental values obtained for the different variables.

In fact, as shown above, *Montecarlo* W flour has a relatively low fAsn content, corresponding to 1.603 ± 0.150 mmol/kg in the flour and to 0.743 ± 0.040 mmol/kg in the dough (Figure 2), but the wood oven baked pizza base obtained with this flour type, showed the highest value of AA, amounting to 1176.45 ± 28.12 μg AA/kg_{dw} of pizza base (Figure 5.4). This behaviour could be due to the high amount in reducing sugars in the obtained dough (4.785 ± 0.397 g/100 g). Nevertheless, the pizza base made with *Stromboli* W flour had an AA content of 1075.78 ± 62.53 $\mu\text{g}/\text{kg}_{\text{dw}}$, thus similar to that of the pizza base made with *Montecarlo* W flour, although *Stromboli* W flour, among all, presented the highest fAsn content (Figure 5.2). The lower formation of acrylamide in *Montecarlo* R, *Sothys* R and *Cosmic* R samples (Figure 5.4) was mainly due to the lower content of fAsn and reducing sugars in flour and dough compared to the other samples (Figure 5.1 and Figure 5.3B), with a good correlation with both variables. However, for the last cited varieties, acrylamide formation, fAsn and reducing sugars content were not correlated between wholemeal and refined flours, except for *Montecarlo* cultivar, indicating that other factors in the flour may influence acrylamide formation in wood oven baked pizza base.¹⁸ Indeed, as also seen in Figure 5.5, for *Sothys* and *Cosmic* W, another parameter affecting the formation of acrylamide was the content and type of fibre in the flour. Moreover, it is known that water absorption is affected by this variable and also the formation of acrylamide depends on the water activity in the dough at baking stage. When the samples present a high fibre content

it is difficult to establish the exact percentage of water for the flour. In this study it was fixed at 61 % of water for all selected wheat varieties, since formed dough after kneading was easy to form for leavening and roll out in a disc shape for baking in a wood oven.

5.3.8 Starch digestibility of pizza base

In vitro starch digestibility of pizza base samples baked in a wood oven, was performed using the enzymatic method, and the results obtained related to the amounts of rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) are shown in Table 5.4.

Table 5.4: *In vitro* digestible starch in pizza obtained with wholemeal (W) and refined (R) flours.

Flour	Cultivar	RDS (%)	SDS	RS	SDRI	RAG
W	<i>Stromboli</i>	49.47±3.47 ^a	14.19±3.73 ^a	0.68±0.05 ^b	76.90±5.76 ^a	54.97±3.85 ^a
	<i>Montecarlo</i>	51.66±3.18 ^a	11.95±1.50 ^a	0.91±0.02 ^a	80.03±2.88 ^a	57.40±3.53 ^a
	<i>Sothys</i>	53.76±2.38 ^a	8.29±0.40 ^b	0.18±0.00 ^c	86.39±0.03 ^b	59.73±2.65 ^a
	<i>Cosmic</i>	55.80±0.54 ^a	8.33±2.55 ^a	0.28±0.06 ^d	86.69±3.46 ^b	62.00±0.60 ^a
R	<i>Stromboli</i>	58.27±0.69 ^{a*}	7.00±1.07 ^{a*}	0.68±0.08 ^a	88.36±1.21 ^{a*}	64.74±0.77 ^{a*}
	<i>Montecarlo</i>	59.16±1.22 ^{a*}	6.32±0.72 ^{a*}	0.93±0.03 ^b	89.09±0.80 ^{a*}	65.73±1.36 ^{a*}
	<i>Sothys</i>	64.08±5.30 ^{a*}	9.56±3.01 ^a	0.66±0.06 ^{a*}	86.17±4.54 ^a	71.20±5.89 ^{a*}
	<i>Cosmic</i>	63.84±2.62 ^{a*}	8.68±0.17 ^a	1.00±0.00 ^{b*}	86.82±0.67 ^a	70.94±2.91 ^{a*}

RDS: after 20 min; SDS: after 120 min; RS: after 240 min. Significance calculated by ANOVA statistical test with Bonferroni ($p < 0.05$). Different lower case letters indicate significant differences between selected wheat cultivars. Significant differences between wholemeal (W) and refined (R) flours are marked by *.

As shown in Table 5.4, RDS, i.e., in 20 min of digestion, occurs in higher amounts for pizza made with refined flours than wholemeal flours. The low value of RDS in pizza samples obtained with wholemeal flours is due to the presence of fibre, the constituents of which, being resistant to hydrolysis by digestive enzymes, are fermented at the colon level by intestinal microflora.⁵⁹ In particular, soluble fibre, at the intestinal level, is capable of absorbing water and forming a gel, which acts as a barrier to the attachment of enzymes to their respective substrates, thus delaying the release of hydrolysis products.⁶⁰ Using the same

method, the SDS, i.e., in 120 min of digestion, in all pizza base samples was also evaluated. In general, it was seen that SDS values were much lower than RDS values, both for pizza made with wholemeal and refined flour. Specifically, the lowest RDS values were found in the samples prepared with the wholemeal flours, *Stromboli* and *Montecarlo*, with values of 49.47 ± 3.47 and 51.66 ± 3.18 %, respectively. Whereas the highest RDS values of 64.98 ± 5.30 and 63.84 ± 2.62 % were found in the doughs prepared with the refined flours, *Sothys* and *Cosmic*, respectively. In contrast, the results show that the SDS values of pizza made with wholemeal wheat flours are higher than those made with refined flours.

This result agrees with the theory that pizza is a food with a high glycemic index; in fact, after baking almost all the starch becomes rapidly digestible and, therefore, easily hydrolysed by intestinal enzymes. The glycemic index is a parameter that describes the potential of foods to increase blood glucose, that is, the rate at which glucose is released into the bloodstream following meal consumption. It has been shown that, the presence of soluble fibre in wholemeal flours modulates the glycemic curve as its viscosity makes it difficult for hydrolytic enzymes to act, slowing gastric emptying and, therefore, the subsequent absorption of glucose.⁶¹

Despite the differences in fibre content between wholemeal and refined flours, numerous studies have shown different opinions on the ability of fibre-rich products to modulate the glycemic curve and given the difficulty of developing *in vivo* tests to assess the glycemic index, *in vitro* methods have been developed over the years to assess the digestibility of these products. To this end, Englyst et al.⁶² proposed the rapidly available glucose index (RAG), which describes, *in vitro*, the amount of glucose released upon ingestion of a food, and it is instrumental in establishing the glycemic index of grain products. The RAG corresponds to the amounts of free sugars and starch, excluding resistant starch, available for intestinal absorption and, therefore, corresponds to the rapidly digestible starch (RDS) fraction. The RAG values for pizza made with wholemeal and refined flours are shown in Figure 5.5. As with the RDS values, the RAG values of the wholemeal wheat flours are lower than those of

the refined flours, and specifically, *Sothys* and *Cosmic* refined flours show higher RAG levels (71.20 and 70.94 %) than the other flours. An additional parameter is the SDRI, or starch digestion rate index, an indicator that describes the digestibility of starch *in vitro* (Ronda et al., 2012).⁶³ The SDRI was obtained by dividing the RDS values by the amount of total starch present in the flour samples. The SDRI values for pizza made with wholemeal and refined flours are shown in Figure 5.5B. It can be seen that the flours obtained with the genotypes *Sothys* and *Cosmic* show a high percentage of SDRI, and no significant differences were found between the respective wholemeal and refined flours. On the contrary, for *Stromboli* and *Montecarlo* genotypes, SDRI values were lower for wholemeal than refined flours, showing significant differences between the samples.

5.3.9 Microstructure of leavened dough

Dough structures analysed by SEM are shown in Figure 5.6. Electronic micrographs showed differences in the structure of the dough obtained from different flours. The micrographs show differences in starch granules, protein network and pore size. The wheat-dough starch granules are spherical or oval in shape and are clearly visible in all samples.⁶⁴ In the details, for the doughs prepared with *Montecarlo* and *Stromboli* wholemeal and refined flours, a greater presence of protein filaments with a fibrous structure was found (Figure 5.6A and 5.6B).

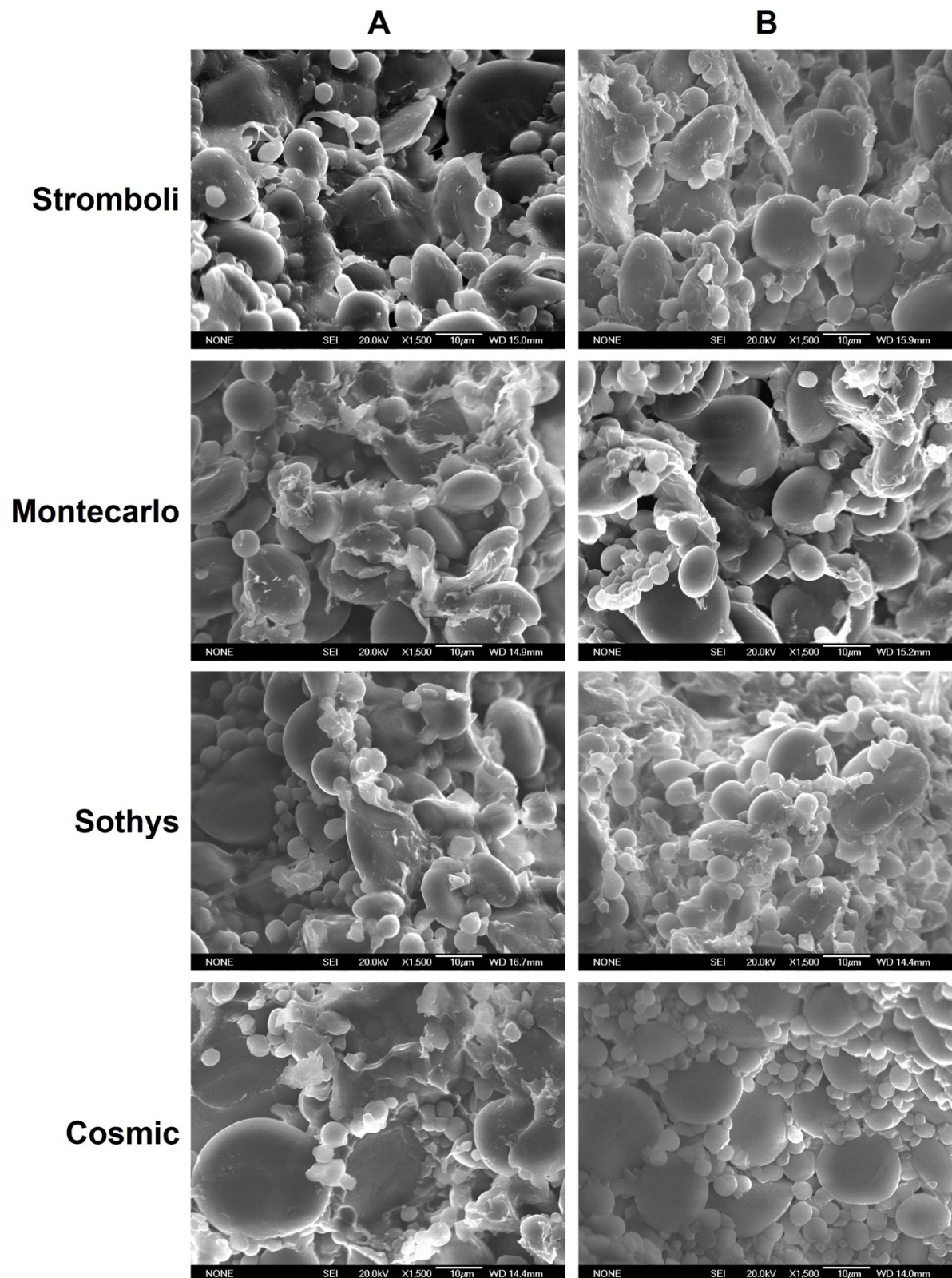


Figure 5.6: SEM images (1500×) of different dough after leavening, prepared with wholemeal (A) and refined (B) flours from selected wheat cultivars.

Moreover, it appears that for *Montecarlo* flour, the morphological structure of the dough after leavening is more open, with medium to small holes, corresponding to air bubbles produced during fermentation, in agreement with the results obtained from the leavening kinetics, in which the sample achieves a higher volumetric index than the other samples, both for wholemeal and refined flours (Figure 5.1). On the contrary, *Cosmic* and *Sothys* samples, show a more compact morphological structure with fewer air bubbles (Figure 5.6C and 5.6D); this result is in agreement with both obtained from the leavening kinetics in which the volumetric index was lower compared with the other two samples (Figure 5.1), and those reported on digestible starch (Table 5.4), in which the rapidly digestible starch (RDS) was higher for both wholemeal and refined compared with the other genotypes. Therefore, this indicates that there is a good correlation between the physical structure of dough and its glycemic index, as reported in previous studies.⁶⁵⁻⁶⁷

5.3 Conclusions

The selected bread wheat varieties showed a different potential in the formation of AA in the final product, pizza in this case, due to the different levels of fAsn present in the respective flours. The lowest levels of AA were found in pizza made from the refined flours, especially from *Montecarlo* and *Sothys* varieties. Furthermore, *in vitro* starch digestibility in prepared pizza showed a higher amount of RDS in pizza prepared with refined flours compared to wholemeal ones. Thus, consumption of products prepared with wholemeal flours could slow the release of glucose into the bloodstream following meal ingestion due to the higher fibre content but, at the same time, their use may result in higher levels of acrylamide in baked foods. This study underlined that not only the fAsn level influences AA formation in baked foods, but also other intrinsic factors of flours such as reducing sugar content, the amount of dietary fibre contained in bran, and different flour absorption indices (WAls). Further studies considering other wheat genotypes with low fAsn content for the preparation of baked foods are needed to confirm the results obtained in this

study. Nevertheless, the study confirms that the use of low fAsn wheat grains is a good strategy to mitigate acrylamide risks in baked wheat derivatives.

Acknowledgments

The authors wish to thank the following Italian companies for providing the bread wheat varieties here used: ISTA Sementi (Lodi), RAGT Italia, Masi Torello, FE, Semetruria (Tarquinia, VT), and Nova Sem (Genola, CN). Moreover, Dr. Chiara Delogu, CREA-DC (Tavazzano, LO, Italy), is kindly acknowledged for providing the official gliadin and glutenin compositions of the four bread wheat varieties. The authors also thank Aniello Falciano for dough preparation and pizza baking in the wood oven.

5.4 Funding

This research was funded by the MIUR, PRIN 2017—2017SFTX3Y—The Neapolitan pizza: processing, distribution, innovation, and environmental aspects.

Part of the research was carried out in the framework of the national program for "Departments of Excellence 2023-27" of MUR (Italian Minister of University and research) at DAFNE, University of Tuscia.

This work was also partially funded by the European Regional Development Fund under the ROP of the Lombardy Region ERDF 2014–2020—Axis I “Strengthen technological research, development and innovation”—Action 1.b.1.3 “Support for co-operative R&D activities to develop new sustainable technologies, products and services”—Call hub.

CONFLICT OF INTEREST

The authors reported no potential conflict of interest.

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Chapter 6. Optimization of a Genome Editing Protocol to reduce free Asparagine content in Durum Wheat seeds (*Triticum turgidum* L. ssp. *Durum* Desf.)

6.1 Introduction

6.1.1 Asparagine metabolism

Asparagine plays an important role in nitrogen transport and acts as a nitrogen storage molecule in plants (Lea et al., 2007).

As reported by Curtis and collaborators (2018a), asparagine metabolism involves a very complex network, composed of 212 genes and about 250 reactions [51].

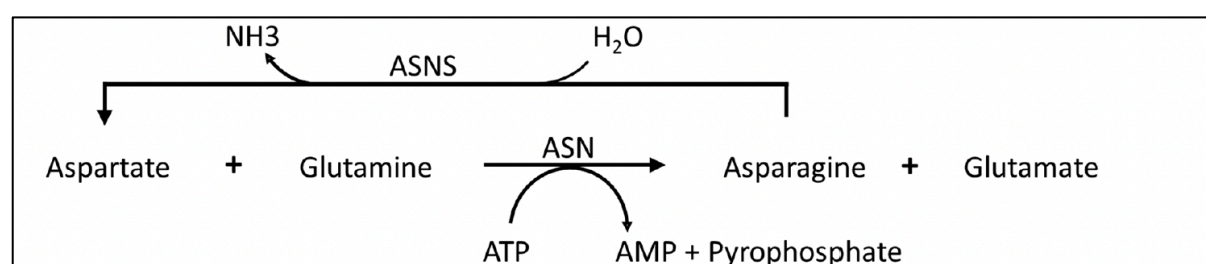


Figure 6.1: schematic representation of basic asparagine metabolic reaction. Figure taken from Oddy J. et al., 2020 [52].

The final step of asparagine synthesis is the reaction catalysed by the enzyme asparagine synthetase (EC:6.3.5.4), which mediates the transfer, in an ATP-dependent manner, of an amino group from glutamine to aspartate [53,54] producing asparagine and glutamate.

In plants, the cloning of an ASN gene was first reported in 1990 by Tsai and Coruzzi, who cloned two cDNAs from pea (*Pisum sativum*) coding for the AS1 and AS2 proteins (Tsai and Coruzzi, 1990).

The number of asparagine synthetase genes in the genome can vary depending on plant species (Lam et al., 1998; Rommens et al., 2008). *Arabidopsis Thaliana* genome contains three asparagine synthetase (Lam et al., 1994; Lam et al., 1998;) genes (*AtASN1*, *AtASN2*, *AtASN3*) which are differentially expressed and regulated by different stimuli, such as light, sucrose,

stress and the supply of organic nitrogen (Rommens et al., 2008). For example, the regulation of *AtASN* genes by bZIP transcription factors in response to sucrose input has been demonstrated (Lam et al., 1998). Under low sucrose levels *AtZIP11* induces the expression of *AtASN* genes, whereas, under high sucrose concentration, others bZIP transcription factors (*AtbZIP9*, *AtbZIP10*, *AtbZIP25*, *AtbZIP63*) down-regulates the *ASN* genes (Hummel et al., 2009). Sucrose nonfermenting-1 (*SNF1*)-related protein kinase-1 (*SnRK1*) is a protein kinase involved in plant-sugar signalling pathway (Hey et al., 2009), which was linked to up-regulation of *AtASN1*, once over-expressed in plant (Baena-González et al., 2007; Baena-González et al., 2008, Confraria et al., 2013). In cereals, the gene families of asparagine synthetase include several *ASN* genes that show a tissue-specific expression regulation (Todd et al., 2008; Duff et al., 2011; Avila-Ospina et al., 2015; Gao et al., 2016; Raffan et al., 2021).

6.1.2 The asparagine synthetase genes family in wheat

Asparagine synthetase gene family has been reviewed recently by Raffan and Halford (2021).

Bread wheat (*Triticum aestivum* L., genomes AABBDD) contains five asparagine synthetase genes per genome: *TaASN1*, *TaASN2*, located on chromosomes 5 and 3, respectively, *TaASN3.1* and *TaASN3.2* located on chromosome 1, and *TaASN4* located on chromosome 4 (Xu et al., 2018). In some varieties, the *TaASN2* gene on chromosome 3B is missing.

Apart from *TaASN4*, which has recently been identified and for which little information is available, *TaASN* genes show differential expression depending on tissues and growth stages (Gao et al., 2016). In particular, *TaASN2* is expressed in both embryo and endosperm, and is the most expressed asparagine synthetase gene during mid/late stages of grain development.

Similarly, durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.), genomes AABB) shows the presence of five *ASN* genes in each of its genomes (Curci et al., 2018).

The first characterisation of asparagine synthetase genes in durum wheat was reported in 2018 by Curci and collaborators who characterised two asparagine synthetase genes (Curci et al., 2018).

More recently, Raffan and Halford identified a total of ten genes coding for asparagine synthetase, five per genome being durum wheat a tetraploid species (Raffan and Halford, 2020).

Through similarity analysis and sequence/location comparison, *TdASN1*, *TdASN2*, *TdASN3.1*, *TdASN3.2*, *TdASN4* were identified and annotated.

TdASN1-A (TRITD5Av1G114980) and *TdASN1-B* (TRITD5Bv1G097460) showed 99.8% similarity with the *TaASN1* gene copies on chromosome 5A and 5B, respectively, of bread wheat.

TdASN2-A (TRITD3Av1G021350) and *TdASN2-B* (TRITD0Uv1G024260) showed a higher similarity to the *TaASN2* genes, with TRITD0Uv1G024260 being slightly more similar to the A genome *TaASN2* than TRITD3Av1G021350 did (95.6% vs. 94.7%). Still, TRITD3Av1G021350 has been assigned to chromosome 3A while TRITD0Uv1G024260, which has not been assigned, presumably represents the gene from chromosome 3B.

In general, from similarity analysis the identity between orthologues in bread and durum wheat ranged between 94.7% to 99.8%, suggesting a high evolutionary conservation of this gene family (Table 6.1).

Table 6.1: ASN genes in *Triticum turgidum* L. ssp. *durum* (Desf.) and percentage identity base on nucleotide sequence with orthologues in *Triticum aestivum* L. cv. Chinese Spring. In this cultivar the *TaASN2* gene in chromosome 3B is missing. ** compared to *TaASN2* in chromosome 3A.

Bread wheat		Durum wheat			
gene name	chr. Location	gene name	chr. Location	identity %	Svevo geneID
TaASN1	5A	TdASN1	5A	99.8	TRITD5Av1G114980
TaASN2	3A	TdASN2	3A	94.7	TRITD3Av1G021350
TaASN3.1	1A	TdASN3.1	1A	98.2	TRITD1Av1G215640
TaASN3.2	1A	TdASN3.2	1A	97.1	TRITD1Av1G222870
TaASN4	4A	TdASN4	4A	96.3	TRITD4Av1G053230
TaASN1	5B	TdASN1	5B	99.8	TRITD5Bv1G097460
TaASN2	missing	TdASN2	Unassigned	96.1**	TRITD0Uv1G024260
TaASN3.1	1B	TdASN3.1	1B	97.7	TRITD1Bv1G207110
TaASN3.2	1B	TdASN3.2	1B	98.8	TRITD1Bv1G220260
TaASN4	4B	TdASN4	4B	99.8	TRITD4Bv1G119970

6.1.3 Reduction of free asparagine accumulation in the grain by CRISPR/Cas9 editing of asparagine synthetase gene *ASN2*

Among the five *TaASN* genes in wheat (*TaASN1*, *TaASN2*, *TaASN3.1*, *TaASN3.2*, *TaASN4*) (Raffan and Halford., 2020), *TaASN2* is the one presenting the highest expression in the grain of wheat plants (Gao et al., 2016; Curtis et al., 2019).

Starting from this data, Raffan and collaborators applied CRISPR/Cas9 technology to knock out *TaASN2* in bread wheat (*Triticum aestivum*) cv. Cadenza (Raffan et al., 2021). In this cultivar, a single copy of the *TaASN2* gene is present in all subgenomes.

Their work reported a significant reduction in free asparagine levels in seeds of T1 plants, with free asparagine reduction up to 90% in those plants where all six *TaASN2* alleles (both alleles in each genome) had been edited.

In addition, they found that the highest reduction in free asparagine concentration in the grain could be observed in when genome A copies (rather than genome B or genome D copies) were edited. In general, plant containing edited genes only in one genome showed a smaller reduction in free asparagine concentration in the grain. In all cases, the free asparagine concentration was still lower than in wild-type. Considering the pool of all free

amino acids, free asparagine showed the highest reduction in the edited plants.

These results indicate that *TdASN2* genes as potential candidate to reduce free asparagine in durum wheat grain via genome editing.

To confirm this prediction, we investigated the expression of *TdASN* gene family in durum wheat.

From a transcriptome analysis (available at CNR-IBBA; unpublished data) carried out on six stages of grain development in cv. Svevo, the transcript abundance related to *TdASN* genes was analysed, confirming that, among all *TdASN* genes, *TdASN2* genes are the ones presenting highest expression during late stages of grain development (Fig.6.2). These data confirmed that *TdASN2* is a good candidate for genome editing to decrease free asparagine accumulation in durum wheat seeds.

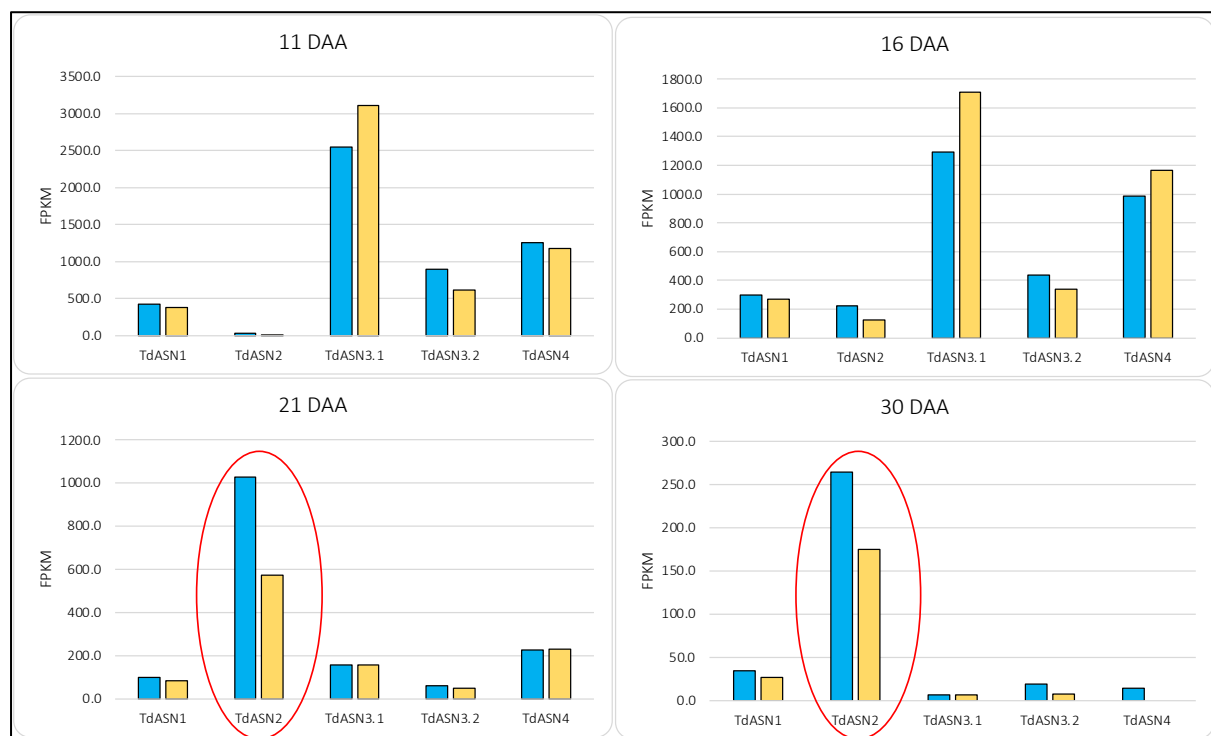


Figure 6.2: Barplot reporting expression levels of *TdASN* genes in four grain developmental stages. Blue bars: *TdASN2*-A; Yellow bars: *TdASN2*-B.

6.2 Material and Methods

6.2.1 gRNA design

To build the final CRISPR/Cas9 vector three gRNAs were utilised. All gRNAs used in this work target the coding sequences of the two genes *TdASN2* (*TRITD3Av1G021350* and *TRITD0Uv1G024260*), present in the genome of the cv. Svevo (Maccaferri et al., 2019). Two of the three gRNAs had been already used by Raffan and Halford (2020) to successfully to knock-out *TaASN2* gene in bread wheat cv. Cadenza. In an effort to increase editing efficiency, a third gRNA was designed targeting a different region in the first exon of *TdASN2* genes. All gRNAs were multiple checked as described below. The gRNAs target the first exon of both *TdASN2* genes in cv. Svevo and should avoid undesired editing in other *ASN* genes.

Blast analysis did not evidence the presence of any likely off-target sequences in the cv. Svevo genome. These gRNAs were also aligned to the cv. Svevo *ASN* genes and scored based on their GC content and out-of-frame scores, a prediction of the probability that mutations induced by the guide RNA will disrupt the open reading frame (Bae et al., 2014). Particular care was taken to monitor possible off target events affecting *TdASN1* genes (on Chromosome 5A and 5B), which are most similar to *TdASN2* genes.

To build the final CRISPR/Cas9 vector three gRNAs were utilised. Two of the three gRNAs were designed and used by Raffan and Halford (2020) and successfully used to knock-out *TaASN2* gene in bread wheat cv. Cadenza. Third gRNA was designed and multiple checked as described above.

6.2.2 Golden Gate assembly of plasmid constructs

The BAR-Cas9 final vector was assembled through three-step Golden Gate reaction using as destination plasmid pICSL22005 (kindly provided by Vladimir Nekrasov, Rothamsted Research). Assembly method was based on protocol described by Hann and collaborators (2020).

The assembly system required multiple step reactions which involves different DNA components to be ligated together in a specific order (fig. 6.7) and,

through three levels of cloning vectors, it is possible to obtain the final vectors with all components necessary for gene editing mediated by CRISPR/Cas9 technology.

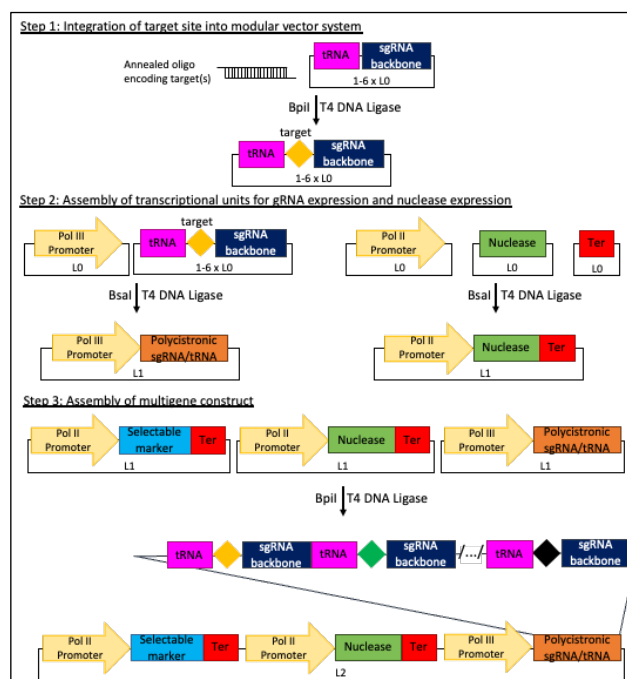


Figure 6.7: A flowchart diagram illustrating assembly of level 2 constructs with gRNAs expressed as tRNA-sgRNA polycistronic units. Figure taken from supplementary material Hahn F. et al (2020) [84].

The gRNAs were synthesised as primer pair (Tab. 6.2), removing PAM sequence and adding a 4 nucleotide (nt) tail (4 nt in red in Table 6.2) corresponding to the restriction site recognised by the restriction enzyme Bpil, which is used for the assembly of gRNAs in level 0 back-bone vectors.

Table 6.2: list of primer used for Golgen Gate cloning of gRNAs used in this study.

Name	Sequence
SvASN2.gRNA1.F	TGCAAGGGGTGCGGCGACGAGTCGC
SvASN2.gRNA1.R	AAACGCGACTCGTCGCCGCACCCC
SvASN2.gRNA2.F	TGCAAGTTGTCGGCGACCTGGTGC
SvASN2.gRNA2.R	AAACGCACCAGGTCGCCGACAAC
SvASN2.gRNA3.F	TGCACTCGCAGTCACTGCCGGTC
SvASN2.gRNA3.R	AAACGACCGGCAGTGACTGCGAGG

General Golden Gate (GG) cut-ligation reaction allows to replace *LacZa* reporter gene included in back-bone vectors with desired gRNA. Due to polycistronic design of the final vectors, three different back-bone vectors were used to insert gRNAs in the correct order in final vectors. At the end of this reaction step, three vectors, each carrying one gRNA, were produced (Tab. 6.3, 1st cloning step).

Table 6.3: List of vectors used for the assembly of the level 2 final vector.

Cloning step	Name	Applicatoin	Addgene ID
1 st	pFH113	level 0 backbone vector (position 1)	#128210
1 st	pAK002	level 0 backbone vector (position 2)	#128215
1 st	pAK003	level 0 backbone vector (position 3)	#128216
2 nd	pFH31	Wheat TaU3p	#125884
2 nd	pAK-EL-003	endlinker	#1000000159
2 nd	pICH47751	level 1 backbone vector	#48002
3 rd	pICSL22005	level 2 backbone vector	unpublished
3 rd	pFH114	ZmUBlp::BAR:NOS† cassette	#128408
3 rd	pICH41766	endlinker	#48018
3 rd	pFH66	SpCas	#131765

A second GG cut-ligation reaction was performed to assemble each generated level 0 tRNA-gRNA modules from the corresponding level 0 vector to a transcription unit carrying the wheat U3 promoter into single level 1 back-bone vector (Tab. 6.3, 2nd cloning step).

Finally, a third GG cut-ligation reaction was performed to obtain the final vector carrying on all DNA fragments necessary for CRISPR/Cas9 machinery (Tab. 6.3, 3rd cloning step). All intermediated and final obtained vectors were sequenced. Nucleotide sequence analysis was performed by Eurofins Genomics (Wolverhampton, UK). The final plasmid used for the bombardment is reported in Figure 6.8.

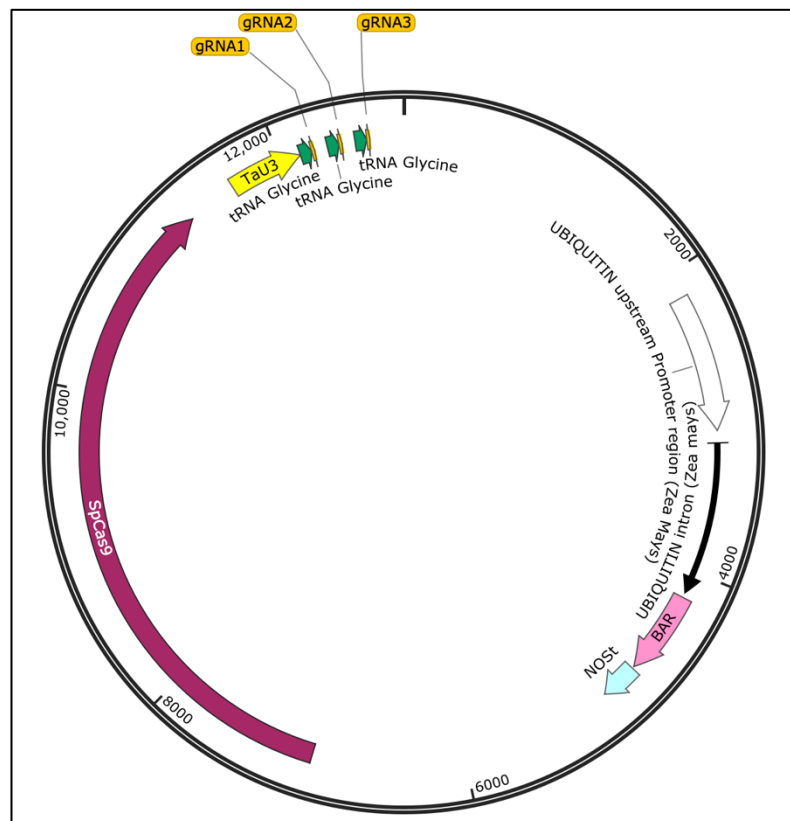


Figure 6.8: Level 2 final vector used for plant transformation with some of the feature highlighted.

6.2.3 Plant growth

Durum wheat seeds of the cv Svevo were germinated on filter paper and then transferred into soil (Rothamsted Prescription mix soil). Different sowings were performed, once per week, to provide constantly available seeds at the correct developmental stage for embryo isolation.

Plants were grown in single pots (25 cm) in a controlled environment at 18 °C/15 °C under a 16 h light/8 h dark photoperiod with a light intensity of 550

$\mu\text{mol m}^{-2} \text{s}^{-1}$ (at 75cm from the ceiling, Osram Fluorescent lamps) and 70/75 % relative humidity.

Svevo spikes were daily checked to visually evaluate the correct developmental stage for embryo isolation (Fig. 6.9). Immature seeds were collected and surface-sterilised in 70% (v/v) ethanol for 2 min, then for 3 min in 0.45% NaOCl (sodium hypochlorite), followed by four rinses in sterile water, before embryos isolation.

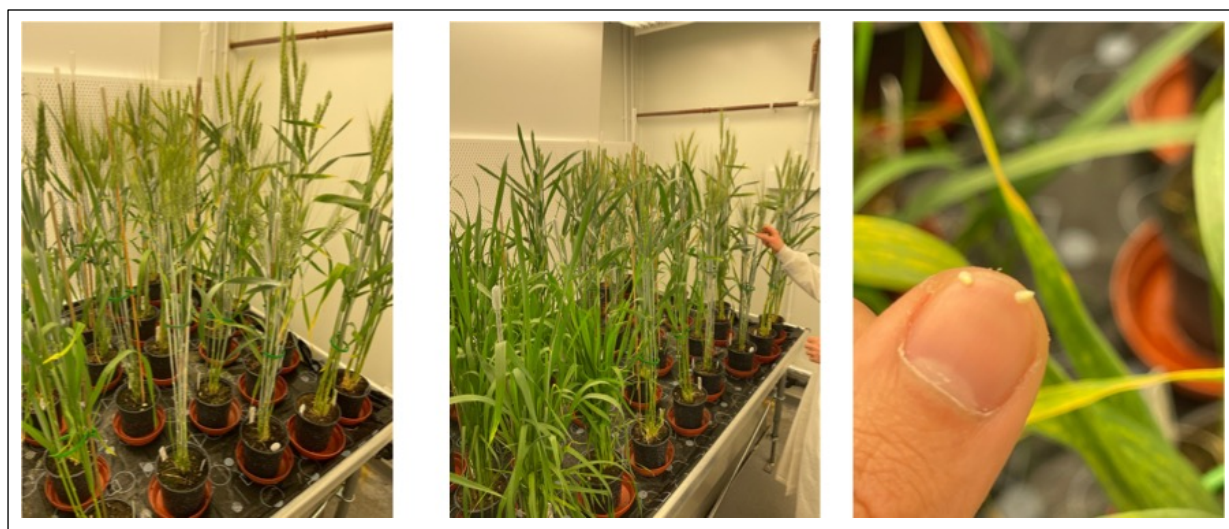


Figure 6.9: Growth chamber and embryos inspection.

6.2.4 Plant Transformation

The transformation of immature embryos of durum wheat plants (cv. Svevo) was performed following the protocol described in volume 2124 of Methods in Molecular Biology (MIMB, 2020, <https://doi.org/10.1007/978-1-0716-0356-7>)^[85] with minor modifications. The final plasmid was transferred into immature embryos through biolistic technology using a Particle Delivery System PDS-1000/He (Bio-Rad). Selected embryos (selected embryos 1.5–2 mm) were placed in the centre of the plate to form a circle with a diameter of 25–30 mm and were bombarded at 700 psi (50 bar). Transformation protocol consisted of a series of steps which induce embryos regeneration via callus-mediated organogenesis of a plant.

Briefly:

Day 1: The selected embryos were 1.5 to 2.2 mm in length and translucent in appearance. Embryonal axis was excised using a scalpel and forceps and embryos placed (30 per plate), scutellum side up, in the centre of a plate containing solid medium MS_{4.5%} (Table 6.5).

Day 2 or 3: Embryos were pre-cultured for 1 or 2 days and then plasmid was transferred into immature embryos through biolistic technology. After bombardment, embryos were let to “dry” for about 1 h and then incubated on MS_{4.5%} medium (Table 6.5) in the dark at 22°C for one week from isolation date.

Day 7: Embryos were transferred to WLS_{P5} (Table 6.6) medium and incubated in the dark at 22°C for two weeks.

Day 21: Embryos were transferred to WLS_{P10} (Table 6.6) medium and incubated in the dark at 22°C for three weeks.

Day 42: Calluses having little visible green bits were transferred to MSRegn_{P5} (Table 6.7) and cultured in the light at 22°C for two weeks.

Day 56: Calluses having green shoots and some small roots were transferred to Rooting_{P5} media (Table 6.8) and cultured in the light for two weeks*.

* It may take longer than two weeks, depending to plant growth.

Day 70: Plants were moved to soil and grown in a containment glasshouse.

Stock Solutions

LS major salts (10×): 19 g/L KNO₃, 16 g/L NH₄NO₃, 4.4 g/L CaCl₂·2H₂O, 3.7 g/L MgSO₄·7H₂O, 1.7 g/L KH₂PO₄.

Ethylenediamine-tetraacetic acid-iron (FeEDTA, 100×): 2.78 g/L FeSO₄·7H₂O, 3.73 g/L ethylenediamine- N, N, N', N'-tetraacetic acid, disodium salt.

LS minor salts (100×): 2.23 g/L MnSO₄·5H₂O, 1.06 g/L ZnSO₄·7H₂O, 620 mg/L H₃BO₃, 83 mg/L KI, 25 mg/L Na₂MoO₄·2H₂O, 2.5 mg/L CuSO₄·5H₂O, 2.5 mg/L CoCl₂·6H₂O.

MS vitamins (100×): 10 g/L myoinositol, 0.2 g/L glycine, 100 mg/L thiamine hydrochloride, 50 mg/L pyridoxine hydrochloride, and 50 mg/L nicotinic acid.

Modified LS vitamins (100×): 10 g/L myoinositol, 100 mg/L thiamine hydrochloride, 50 mg/L pyridoxine hydrochloride, and 50 mg/L nicotinic acid.

2,4-Dichlorophenoxyacetic acid (2,4-D, 100 mg/l): 1N NaOH dropwise to 100 mg 2,4-dichlorophenoxyacetic acid until completely dissolved. Make up to 1,000 ml with distilled water.

Picloram (100 mg/l): 1N NaOH dropwise to 100 mg picloram until completely dissolved. Make up to 1000 ml with distilled water.

Zeatin (100 mg/l): 1N NaOH dropwise to 100 mg zeatin until completely dissolved. Make up to 1000 ml with distilled water.

Indole-3-butyric acid (IBA, 100 mg/l): 1N NaOH dropwise to 100 mg indole-3-butyric acid until completely dissolved. Make up to 1000 ml with distilled water.

6-Benzylaminopurine (6BA, 100 mg/l): 1N NaOH dropwise to 100 mg 6-benzylaminopurine until completely dissolved. Make up to 1000 ml with distilled water.

Phosphinothricin (PPT, 20 mg/ml): 400 mg phosphinothricin in 20 ml of distilled water. Sterilise with a 0.22 µm cellulose acetate filter.

Table 6.4: MS_{4.5%S} medium composition.

MS _{4.5%S}		
Component	Concentration	Quantity
Agargel	10g/L	500 ml/L
2X MSAA+4.5%S	2X	500 ml/L
AgNO ₃	20ug/ul	500 ul/L
2,4-D	1ug/ul	500 ul/L

Table 6.5: WLS_{P5/P10} medium composition.

WLS _{P5/P10}		
Component	Concentration	Quantity
LS major salts	(10×)	100 ml/L
FeEDTA	(100×)	10 ml/L
LS minor salts	(100×)	10 ml/L
MS vitamins	(100×)	10 ml/L
glutamine	/	500 mg/L
Casein Hydrolysate	/	100 mg/L
MgCl ₂ ·6H ₂ O	/	750 mg/L
Maltose	/	40 g/L
MES	/	1.95 g/L
Ascorbic acid	(100mg/ml)	1 ml/L
AgNO ₃	(20mg/ml)	42.5 ul/L
2,4-D	(1mg/ml)	500 ul/L
Picloram	(5mg/ml)	440 ul/L
Agarose	(10g/L)	500 ml/L
PPT	(10mg/ml)	500 ul/L (WLS _{P5}) or 800ul (WLS _{P10})

Table 6.6: MSRegn_{P5} medium composition.

MSRegn _{P5}		
Component	Concentration	Quantity
MS salts (M0221)	/	4.3 g/L
Casein hydrolysate	/	1 g/L
Maltose	/	30 g/L
BCI vitamins	(100×)	10 ml/L
CuSO ₄	(25mg/ml (0.1M))	1.245 mg/L
Zeatin (10mg/ml)	(10 mg/ml stock)	2.5 mg/L
PPT	(10mg/ml)	500 ul/L
Agarose (separately autoclaved)	(10g/L)	500 ml/L

Table 6.7: Rooting_{P5} medium composition.

Rooting _{P5}		
Component	Concentration	Quantity
LS major salts	(10×)	100 ml/L
Ferrous sulphate chelate solution	/	10 ml/L
LS minor salts	(100×)	10 ml/L
Modified LS vitamins	(100×)	10 ml/L
Sucrose	/	15 g/L
MES	/	0.5 g/L
Gelrite	/	3 g/L
IBA	100 mg/ml	2 ml/L
PPT	(10mg/ml)	500 ul/L (Rooting _{P5})

Rothamsted Prescription mix soil (Petersfield products, UK): 75% medium grade peat, 12% sterilized loam, 3% medium grade vermiculite, 10% grit (5 mm screen, lime-free), 3.5 kg Osmocote Exact 3/4 month/m³ (slow-release fertilizer, 16N/11P/11K plus micronutrients, supplied by Scotts UK Professional, Ipswich, Suffolk), 0.5 kg of PG mix/m³ (14N/16P/18K granular fertilizer plus micronutrients, supplied by Hydro Agri (UK), Ltd., Lincs.), lime ~3 kg to pH 5.5–6.0, Vitax Ultrawet at 200 mL/m³ (wetting agent).

6.3 Results and Discussion

To generate durum wheat (cv. Svevo) plants lacking in *TdASN2* gene function, about 700 immature embryos (including control embryos not bombarded) were bombarded in seven different experiments.

Out of the 700 embryos, seven T0 plants (from three different experiments) were regenerated and grown in containment glasshouse until complete seeds maturity.

Seedlings were grown from the T1 seeds of regenerated T0 plants. DNA from 25 potentially edited T1 plants were then analysed through PCR using the primers UbiPro4 (5'-TTTAGCCCTGCCTTCATACG) and Bar2 (5'-GAAGTCCAGCTGCCAGAAAC) to amplify the BAR gene used for the selection.

Among the 25 analysed plants, two transformed plants were found (Figure 6.10).

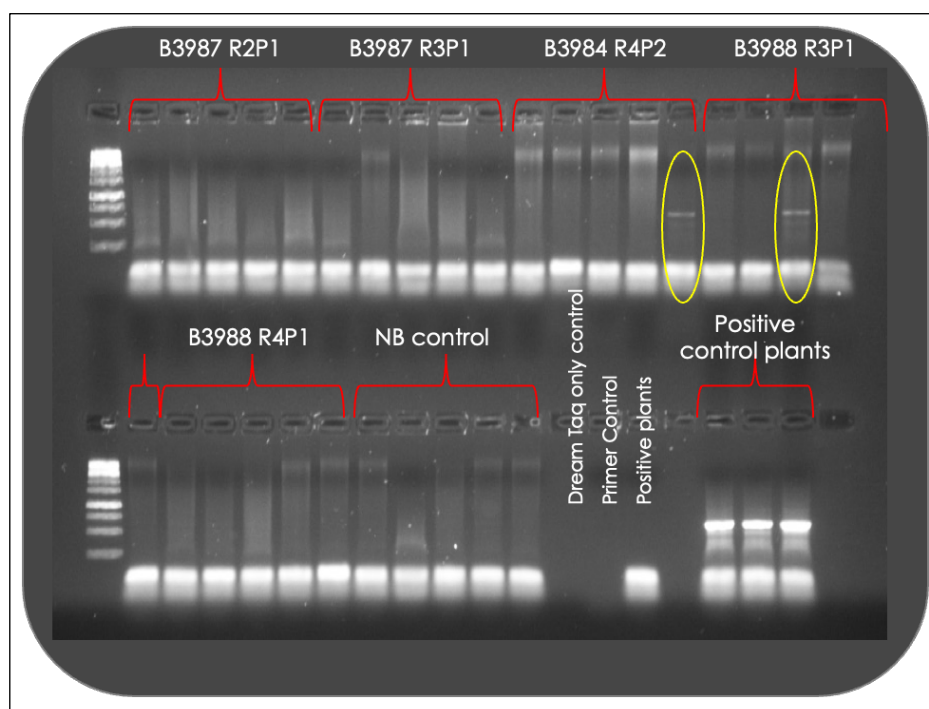


Figure 6.8: screening of T1 plants by PCR.

The following step will consist in the analysis of the two transformed plants to assess the editing in *TdASN2* gene. To this purpose, a PCR will be performed

using the primers ASN2ge-F1 (5'-GTAGAGCCAAGCCATTCCTG-3') and ASN2ge-R1 (5'-GCGATGACCTCGCAGTCAC-3') to amplify TdASN2 first exon. PCR products will be analysed by the T7 endonuclease I (T7EI) assay (New England Biolabs, Massachusetts, US). If editing will be confirmed, edited plants from T7EI assay will be sequenced to better characterise modification introduced in the *TdASN2* genes.

The transformation of immature embryos through the biolistic system is being repeated with the goal of eventually obtaining at least three-four independent edited lines.

6.4 References

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Chapter 7. Conclusions and Perspectives

This PhD project aimed to study natural variation in free asparagine levels in durum (*Triticum turgidum* L. ssp. *durum* (Desf.)) and bread (*Triticum aestivum* L.) wheat grains, and to identify candidate genes involved in free asparagine accumulation in wheat seeds.

To our knowledge, this thesis presented the first genome wide association study (GWAS) investigating free asparagine content in durum wheat seeds, as well as the first analysis of free asparagine content in bread wheat elite varieties relevant for the Italian market. Both the GWAS study and the analysis of bread wheat elite varieties provide useful information which can be immediately applied in food production processes and/or could be the basis for further studies on the mechanisms controlling free asparagine accumulation in wheat.

Regarding the durum wheat GWAS, this study utilised a panel of durum wheat landraces extracted from an international durum wheat collection. Wheat landraces are an essential germplasm resource to be utilised as a reservoir of crop genetic diversity, and the aim of this work was to exploit this genetic diversity to identify QTNs associated to free asparagine accumulation. This, together with the utilisation of a mGWAS allowed to identify six QTNs and a set of candidate genes which could play a role in asparagine metabolism and accumulation in developing seeds.

Multiple factors could control free asparagine levels in mature caryopsis. During seed development amino acids are translocated from vegetative tissues to the seed, largely in the form of glutamine, where they can be interconverted, and incorporated into storage proteins (Zhong et al., 2020). Thus the balance between amino acid uptake, interconversion and incorporation into proteins is bound to affect the levels of free amino acids, including asparagine, in the developing and the mature grain.

The results could provide a starting point for functional characterisation of genes potentially involved in controlling free asparagine levels throughout seed development and highlight regulatory steps that may also be responsible for the observed dependence of free asparagine levels on environmental factors such as mineral nutrition and pathogen attack.

Regarding bread wheat characterization, this thesis studied elite varieties relevant for the Italian market, that have not been characterized before with regard to this trait. The analysis of free asparagine accumulation in 54 bread wheat elite varieties identified genotypes showing contrasting free asparagine accumulation (High vs. Low) over the different years and environments. Although the results obtained will have to be validated in further field trials, these selected genotypes could be used as starting material for food production to mitigate the risk of acrylamide in wheat derivatives, thus avoiding the application of other methods, such as the use of asparaginase, which are not suitable to all foods, being expensive or having an effect on product quality. For instance, the study conducted in collaboration with the University of Naples Federico II and the University of Tuscia (Covino et al., 2023, Chapter 5 of the thesis) benefited from the genotypes identified as contrasting free asparagine accumulators in the frame of this thesis (Tafari et al., 2023, Chapter 4 of the thesis). These genotypes have been used to assess acrylamide-forming potential in oven-bake pizza prepared with Neapolitan recipe, a milestone of Italian cuisine.

In addition, the identified genotypes will be used to generate a segregating population (High X Low accumulators) within the collaboration with Società Italiana Sementi. This population will represent a very useful material for further studies on free asparagine accumulation in wheat grain.

Finally, the optimization of a protocol to transform and editing of durum wheat cv. Svevo should allow us to generate a low-free asparagine phenotype plants in a cultivar of great economic interest. This will also allow to exploit genomic

information available for the v. Svevo to study the impact of *TdASN2* gene inactivation on overall gene expression and metabolism, thus providing a driving force for studies on free asparagine accumulation.

In conclusion, this thesis presents an initial characterization of wheat genetic materials that have not been characterized before for free asparagine content, as well as novel information on the genetics of free asparagine accumulation in wheat grain. This work represents a starting point for further studies on the genetic factors controlling amino acid metabolism in durum wheat seeds and for the generation of lines with low acrylamide forming potential that should be of interest for the food industry worldwide.

Acknowledgments

“This PhD work was supported by the National Research Council program for Industrial Doctorates and by S.I.S. Società Italiana Sementi”

Chapter 8. List of the PhD Activities Done During the Course

8.1 List of Publications

Tafari A., Zuccaro M., Ravaglia S., Pirona R., Masci S., Sestili F., Lafiandra D., Ceriotti A., Baldoni E., 2023. Exploring Variability of Free Asparagine Content in the Grain of Bread Wheat (*Triticum aestivum* L.) Varieties Cultivated in Italy to Reduce Acrylamide-Forming Potential. *Plants* 12, 1349.
<https://doi.org/10.3390/plants12061349> .

Covino C., **Tafari A.**, Sorrentino A., Masci S., Baldoni E., Sestili F., Villalonga R., Masi P., 2023. Mitigation of acrylamide formation in wood oven baked pizza base using wheat grain with low free asparagine content. *Journal of the Science of Food and Agriculture* ([submitted](#)).

Tafari A., Pirona R., Ceriotti A., Zuccaro M., Ravaglia S., Giordano M., Cagliani L. R., Consonni R., Fricano A., Mazzucottelli E., Baldoni E., 2023. Genome Wide Association Study of Free Asparagine Content in Durum Wheat (*Triticum turgidum* L. ssp. *Durum* Desf.) Grain. ([In preparation](#)).

8.2 List of Poster Presentations and Oral Communications

Poster communication at Plant Biology Europe 2021:

Tafari A., Pirona R., Ceriotti A., Ravaglia S., Giordano M., Cagliani L. R., Consonni R., Gasser M., Thomas A., Gilardi F., Rhogayeh N., Mazzucotelli E., Baldoni E. "Exploring variability in free asparagine content to reduce acrylamide-forming potential of durum wheat seeds". 28th June-1st July 2021 On-line congress.

Poster communication at 64th SIGA Annual Congress:

Pirona R., **Tafari A.**, Accotto G. P., Fricano A., Ravaglia S., Giordano M., Marian D., Matic S., Marra M., Vaira A. M., Mazzucotelli E., Rhogayeh N., Ceriotti A., Baldoni E. "Exploiting the potential of Global Durum Panel for plant and human health". 14th-16th September 2021. On-line congress.

Poster communication at 64th SIGA Annual Congress:

Cominelli E., Baldoni E., Bernasconi A., Bernasconi M., Cagliani L. R., Capobianco A., Ceriotti A., Consonni R., Dougué K. R., Ferron L., Galasso I., Genga A., Libanore M., Liberatore C., Locatelli F., Mattana M., Pappalardo V., Pedrazzini E., Pirona R., Pozzo S., Ravasio N., Spatola F., **Tafari A.**, Zaccheria F., Zuccaro M., Sparvoli F. "sPATIALS3 project: improvement of primary productions for a healthier diet". 14th-16th September 2021. On-line congress.

Poster communication at New Phytologist "Next generation scientists 2022":

Tafari A., Pirona R., Ceriotti A., Zuccaro M., Ravaglia S., Giordano M., Cagliani L.R., Consonni R., Fricano A., Mazzucottelli E., Baldoni E. "Genome wide association study of free asparagine content in durum wheat grain". 19th-22nd July 2022, Delta Centre, Tartu, Estonia.

Poster communication at 65th SIGA Annual Congress:

Tafari A., Pirona R., Ceriotti A., Zuccaro M., Ravaglia S., Giordano M., Cagliani L. R., Consonni R., Fricano A., Mazzucottelli E., Baldoni E. "Genome wide association study of free asparagine content in durum wheat grain". **Awarded with one of the prizes for best posters communications.** 6th-9th September 2022, Università Cattolica del Sacro Cuore, Piacenza, Italy.

Poster communication at 4th From Seed to Pasta congress:

Pirona R., **Tafari A.**, Marra M., Fricano A., Ravaglia S., Mazzucotelli E., Matic S., Accotto G., Ceriotti A., Baldoni E. "Exploiting the potential of Global Durum Panel for plant and human health". 26th-28th October 2022, Conference Center Emilia-Romagna Region, Bologna, Italy.

Oral communication at "Acrylamide reduction in foods, from plant breeding to food processing" Congress:

Tafari A., Pirona R., Ceriotti A., Zuccaro M., Ravaglia S., Giordano M., Cagliani L. R., Consonni R., Fricano A., Mazzucottelli E., Baldoni E. "Genome wide association study of free asparagine content in durum wheat grain". 23rd-24th May 2022; Discovery Park, Kent, UK.

Oral communication at "Acrylamide reduction in foods, from plant breeding to food processing" Congress:

Zuccaro M., **Tafari A.**, Ceriotti A., Ravaglia S., Masci S., Sestili F., Lafiandra D., Messia M.C., Covino C., Sorrentino A., Masi P., Baldoni E. "Exploring variability in free asparagine seed content in bread wheat (*Triticum aestivum* L.) elite varieties to reduce acrylamide-forming potential".

23rd-24th May 2022; Discovery Park, Kent, UK.

8.3 List of workshop and course participations

Participation in seminars, courses, conferences, workshop, schools	Title	Location	Date, hours or days
Training course	"Genomic-selection assisted of Wheat"	Bologna (BO)	28 th -29 th October 2019
Annual Meeting	"CREA-GB IV Annual Meeting"	Fiorenzuola d'Arda (PC)	16 th -17 th -18 th December 2019
UniMi course	"Novel strategies to accelerate crop improvement"	Milan (MI)	January 28 th - February 13 th 2020
CNR-IBBA project meeting	"sPATIAL ³ presentation conference"	Milan (MI)	4 th February 2020
UniTus course	"Genetica e fisiologia della produzione"	On-line	8 th -12 th June 2020
UniTus course	"introduzione alla statistica descrittiva"	On-line	8 th -11 th June 2020
SIGA meeting	"SIGA Young Web Meeting"	On-line	7 th July 2020
UniTus course	"Meccanismi di difesa delle piante"	On-line	9 th - 12 th July 2020
UniTus course	"Disegni sperimentali"	On-line	13 th - 15 th July 2020
UniTus course	"Principi di scrittura scientifica"	On-line	13 th - 15 th July 2020
Congress	Virtual Durum Meetings (VDMs) 1 st edition	On-line	27 th - 28 th July 2020
Meeting	"SIGA Commemorative Online Congress"	On-line	23 rd September 2020
Meeting	"SURF Kick-off Meeting"	On-line	29 th September 2020
Webinar	"Horizon Europe: strumento di attuazione di obiettivi politici, scientifici e strategici dell'UE – novità e opportunità per la rete scientifica"	On-line	29 th April 2021
UniTus course	"Plagas emergentes de interés para la agricultura en la UE"	On-line	October - December 2020
Congress	Virtual Durum Meetings (VDMs) 2 nd edition	On-line	30 th November- 1 st December 2020
Web symposium	"Web symposium on plant health"	On-line	16 th December 2020
Web workshop	"Young scientists for plant health"	On-line	16 th December 2020
Webinar	"Innovazione tecnologica e ruolo della ricerca pubblica nel settore sementiero italiano: il caso del frumento duro"	On-line	19 th February 2021

Webinar	"Efforts to obtain cereals and/or pseudocereals with better performance, grain composition or quality"	On-line	4 th March 2021
Webinar	Polyphenol oxidase genes as integral part of the evolutionary history of domesticated tetraploid wheat"	On-line	17 th March 2021
UniTus course	"Statistica Base con R"	On-line	24 th -27 th May 2021
UniTus course	"Statistica Avanzata con R"	On-line	31 st May- 4 th June 2021
UniTus course	"Tecniche molecolari innovative per lo studio del microbioma del latte e del formaggio"	On-line	15 th -27 th June 2021
UniTus course	"Approccio metabolomico per la caratterizzazione e la valorizzazione dei prodotti agroalimentari"	On-line	14 th -22 nd June 2021
UniTus course	"Determinazione della sequenza aminoacidica utilizzando la spettrometria di massa"	On-line	14 th -22 nd June 2021
UniTus course	"Tecniche di ingegneria genetica e proteica abbinate alle produzioni animali e vegetali"	On-line	17 th -23 rd June 2021
Congress	"Plant Biology Europe 2021" Virtual Conference	On-line	28 th June – 1 st July 2021
Congress	Virtual Durum Meetings (VDMs) 3 rd edition	On-line	20 th -21 st July 2021
Congress	"LXIV Convegno annuale SIGA"	On-line	14 th -16 th September 2021
CNR-IBBA project meeting	"sPATIAL ³ WP1 Meeting"	Milan (MI)	29 th September 2021
Workshop	"Virtual Ensembl Plants Browser Workshop"	On-line	2nd-3rd-4th November 2021
Workshop	"Virtual Ensembl Plants REST API course"	On-line	9th-10th-11th November 2021
Webinar	"30 years of Genetic Mapping in Rice - Science, Sustainability and Sanity BBSRC Institutional Sponsorship Fund Seminar"	On-line	17th February 2022
Symposium	"Rothamsted PhD symposium"	Rothamsted Conference hall (Rothamsted Research, England,UK)	1st-2nd March 2022
Workshop	AAB-PlantED training school on communicating the science of GE crops	Rothamsted Conference hall (Rothamsted Research, England,UK)	28th-29th April 2022
Congress	Acrylamide reduction in foods, from plant breeding to food processing	Discovery Park, Kent, UK	23rd-24th May 2022

Workshop	PlantEd WG1 Online Lecture Series	On-line	25th May 2022
UniTus course	"Corso Metodologie avanzate applicate ai processi di trasformazione alimentare"	On-line	6th-9th June 2022
Workshop	"Biotecnologie e adempimenti normativi - Le Neuroscienze applicate alla Sicurezza"	Milan (MI)	22nd June 2022
Workshop	Wheat Genetic and genomics Workshop	Fiorenzuola d'Arda (PC)	23rd-24th June 2022
Congress	"LXIV Convegno annuale SIGA"	Piacenza (PC)	6th-9th September 2022
Webinar series	Webinar dedicated to the COST action: ACRYRED	On-line	15th September 2022
CNR-IBBA course	Statistical learning and deep learning: theoretical background and hands-on sessions.	Milan (MI)	30th-31st January 2023

8.4 Tutorship activities

Tutoring of a bachelor's degree internship (Marco Libanore, University of Milano Bicocca).

8.5 Participations in research project

sPATIALS3: Miglioramento delle produzioni agroalimentari e tecnologie innovative per un'alimentazione più sana, sicura e sostenibile"

Call: "CALL HUB RICERCA E INNOVAZIONE" nell'ambito POR FESR 2014 2020 di Regione Lombardia.

Period: 01/02/2020 - 31/07/2022

Funding: Regione Lombardia

8.6 Abroad internship

Rothamsted Research (Harpenden, Hertfordshire, AL5 2JQ) at Prof. Nigel G. Halford group (January 2022-May 2022).

The aim of the visit was to get trained in wheat genome editing, and to set up a protocol to silence the *ASN2* genes in durum wheat using the CRISPR/Cas9 genome editing system to reduce free asparagine accumulation specifically in the grains, without affecting physiological functions of the plant.