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Agronomic, genetic and biotechnological approaches to lower free asparagine content in cereals: state of the art and perspectives☆

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A B S T R A C T

Acrylamide represents a risk to human health due to its neurotoxicity and genotoxicity. Acrylamide is formed in a wide range of plant-derived foods during normal high-temperature cooking, and industrial processing at more than 120°C and low humidity. The main chemical process that causes the formation of acrylamide is the "Maillard cascade reactions", which involve reducing sugars and free amino acids that are naturally present in many foods. Free asparagine content is the key factor in acrylamide formation in cereal-derived products. Therefore, breeding for low-asparagine varieties could help to minimize acrylamide-associated risks. In this review, we summarize the state of the art about the agronomic, genetic and biotechnological approaches used so far for decreasing free asparagine levels in cereal grain and propose possible strategies that could be exploited in the future to reach this goal.

1. Introduction

1.1. Acrylamide in foods

Acrylamide (C_3H_5NO) represents a risk to human health due to its neurotoxicity and genotoxicity. Acrylamide is a white, odourless and water-soluble molecule, which is rapidly absorbed through the skin and from the gastro-intestinal tract. Once metabolised in the body, a derivative called glycidamide ($C_3H_5NO_2$) is produced, which in turn forms other derivatives capable of binding to DNA both *in vitro* and *in vivo* in animal and human tissues (EFSA, 2015). Acrylamide is classified as a Group 2A carcinogen, defined as "probable human carcinogens" by the International Agency for Research on Cancer (IARC; International Agency for Research on Cancer, 1994). Furthermore, several studies have highlighted that acrylamide can be genotoxic, neurotoxic and toxic to the reproductive system (Chapin et al., 1995; Lopachin, 2004).

Acrylamide is formed in a wide range of foods during normal high-temperature cooking (frying, roasting, baking and grilling), and industrial processing at more than 120°C and low humidity (Tareke et al., 2002). The formation of acrylamide occurs principally in plant-derived foods, whereas acrylamide levels measured in animal-derived foods are very low and do not represent a risk for human health (Tareke et al., 2002). The most important food groups that contribute to acrylamide exposure are fried potato products, coffee, biscuits, crackers, breakfast cereals, crispbreads and soft breads. Cereal-derived products strongly contribute to the intake of acrylamide among the human population, due to the daily quantity and frequency of consumption of these foods, as reported by the EFSA CONTAM Panel (EFSA, 2015). For this reason, there is a strong interest in developing strategies to lower the acrylamide-forming potential in cereal grain. The Food and Agriculture Organization of the United Nations (FAO) and the European Food Safety Authority (EFSA) have analysed various products available on the market and have published related guidelines to minimize the risks related to acrylamide (EFSA, 2015; FAO, 2009). The World Health

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Organization (WHO) has set the maximum level of acrylamide in drinking water at 0.5 μg/L (World Health Organization, 1993); however, foods such as crisps (US chips), baked crisps and crisp bread may contain acrylamide up to 1000 μg/kg and more. Furthermore, in 2011 a joint FAO/WHO expert committee on food additives established a maximum limit of daily consumption of 180 μg/kg body weight (b.w.), to reduce the risk of cancer development, based on data from dose-response models that evaluated the effects of acrylamide exposure in laboratory animals (World Health Organization, 2011). In the European Union (EU), EFSA lowered the threshold to 170 μg/kg b.w. per day and clarified that it is not a "recommended value", but a reference point for calculating the margin of exposure (MOE) (EFSA, 2015). Furthermore, these agencies recommend dedicating great efforts to the development and implementation of mitigation methods for acrylamide in foods, to reduce the levels of this substance as much as possible, given its probable carcinogenic effects (World Health Organization, 2011) (Reg EU, 2017/2158).

Based on this evidence, Benchmark Levels (BLs) have been defined by the European Commission (EC) for the acrylamide content in some foods: 50–800 μg/kg for baked foods, 750 μg/kg for potato crisps and 400 μg/kg for roast coffee (European Union Regulation (EU) 2017/2158). Recent studies confirmed the effectiveness of BL to lower acrylamide content in some foods. Indeed, a general downward trend in acrylamide content was observed from 2018, indicating that specific initiatives of industry can have an effective role in reducing contaminant levels. However, some products still contained acrylamide above the BL (Mesías et al., 2025). Currently, the European Commission is considering reducing BLs and imposing Maximum Levels (ML) for some foods, including all major cereal products (https://ec.europa.eu/food/safety/chemical-safety/contaminants/catalogue/acrylamide_en). Therefore, the scientific world focuses on the development of further mitigation strategies for acrylamide in cooked foods, especially those derived from tubers, storage roots, beans and grains.

1.2. The role of asparagine in the formation of acrylamide in foods

The main chemical process that causes the formation of acrylamide is known as "Maillard cascade reactions"; the same process gives the fried/toasted appearance to foods and makes them tastier (Mottram et al., 2002; Stadler et al., 2002). Maillard reactions involve reducing sugars, such as glucose, fructose or maltose, and free (soluble, non-protein) amino acids, which are naturally present in many foods. Acrylamide forms when free asparagine ($C_4H_8N_2O_3$) participates in the later stages of the reaction. Over the past twenty years, several studies have described strategies to reduce acrylamide formation in foods, such as minimizing the content of reducing sugars and asparagine, increasing moisture, heating at a lower temperature, and reducing processing time (Anese et al., 2010). Since colour forms from similar pathways to acrylamide, it is now common to use colour as a quality control measure, particularly in potato crisp manufacture.

In cereal grain-based foods, free asparagine concentration is the rate-limiting factor for acrylamide formation (Raffan & Halford, 2019). Indeed, the addition of asparagine markedly increases the acrylamide content of bread (Surdyk et al., 2004). A correlation between free asparagine concentration in wheat flour and acrylamide formation after heating has been demonstrated (Raffan & Halford, 2019). Wheat flour is a highly starchy matrix and has a very variable free asparagine content, between 74 and 664 mg/kg of flour (which corresponds to 0.56 – 5.03 mmol/kg; (Claus et al., 2006; Lea et al., 2007; Curtis, Bo, Tucker and Halford, 2018; Navrotskyi et al., 2018). In some cases, the levels can be even higher (Corol et al., 2016). Wholemeal flours contain much higher quantities than refined flours, since in grain free asparagine is mainly stored in germ (embryo) and the external layers that form the bran. Consequently, the acrylamide forming potential is higher in cereal foods derived from wholemeal flour (Covino et al., 2024). In the European Regulation (2017/2158), one of the strategies to reduce the formation of acrylamide is the elimination of free asparagine. There are several strategies aimed at this purpose, one of which concerns the use of the enzyme L-asparaginase, which catalyses the hydrolysis of asparagine into aspartic acid and ammonium (Covino et al., 2023). Another strategy for reducing free asparagine in flours is the use of fermentations that use specific microorganisms to reduce the levels of asparagine or reducing sugars in the dough (Bartkiene et al., 2013; Nachi et al., 2018; Wang et al., 2017). The use of fermentations with lactic acid bacteria or the use of sourdough are possible strategies which, in addition to decreasing the level of acrylamide in baked products, improve some of their nutritional properties (Nachi et al., 2018).

These strategies are undoubtedly useful. Nevertheless, they are not feasible for all food matrices and for all processes and difficulties may occur in their implementation. For this reason, an important recommendation for the food industry is the use of flours (and therefore grains) characterized by a low content of free asparagine. In this context, breeding aimed to create low-asparagine varieties could minimize acrylamide-associated risks. The request of new cereal varieties from food industry could intensify if the EU imposes MLs for acrylamide in cereal foods (Kaur & Halford, 2023).

Free amino acids represent approximately 5% or less of the total nitrogen (N) content of the cereal grain, with asparagine representing 10% or less of the total free amino acids (Lea et al., 2007). Consequently, both scientific research and breeding activities have shown little interest in the past regarding the levels of free asparagine in grains, until the demonstration that this amino acid is a precursor of acrylamide during cooking. For this reason, in recent years an increasing number of studies aimed at identifying specific factors that influence this trait. These studies have shown that free asparagine accumulation in wheat grains varies highly among genotypes (Claus et al., 2006; Corol et al., 2016; Navrotskyi et al., 2018; Curtis, Bo, Tucker and Halford, 2018; Tafuri et al., 2023). It is also well proven that this trait is strongly influenced by environmental and agronomic factors, such as abiotic and biotic stresses (Lea et al., 2007), sulphur (S) and/or N availability (Curtis et al., 2016; Martinek et al., 2009; Wilson et al., 2020), and poor disease control (Curtis et al., 2016; Martinek et al., 2009). The accumulation of free asparagine in wheat grains is a specific genetic trait, and related studies reported low or medium heritability values for this trait (Corol et al., 2016; Emebiri, 2014; Rapp et al., 2018; Tafuri et al., 2025). The interaction of genotype and environmental factors (G × E) is also known to influence free asparagine content in cereal grains (Xie et al., 2021; Postles et al., 2013; Corol et al., 2016; Curtis, Bo, Tucker and Halford, 2018). Therefore, both agronomy and genetics can play an important role to breed and select cultivars that accumulate low and stable free asparagine levels in different growing environments, to mitigate the risk of acrylamide formation in cereal-based products.

The working group 2 (WG2) "Agronomy and plant breeding" of the COST ACRYRED Action (CA21149) "Reducing Acrylamide Exposure of Consumers by a Cereals Supply-chain Approach Targeting Asparagine" aims to assess diversity of free asparagine in cereal species, considering both traditional and commercial varieties, and to identify the genotypes displaying low content of free asparagine in the grain. Further aims are related to understanding the biochemical pathways for free asparagine accumulation and identifying efficient ways for free asparagine reduction in cereal grains, as well as developing fast techniques for screening free asparagine levels in cereals. Providing recommendations for breeders, farmers and food industry is a further target of WG2. In this context, the aim of this review, the authors of which belong to ACRYRED WG2, is gathering information about the potential for decreasing free asparagine levels in the grain of cereals, considering all the factors that influence this trait, from modification of soil composition through fertilization to the use of biotechnology through genome editing (Figure 1). We also present possible strategies that can be exploited in the future to reach this goal.

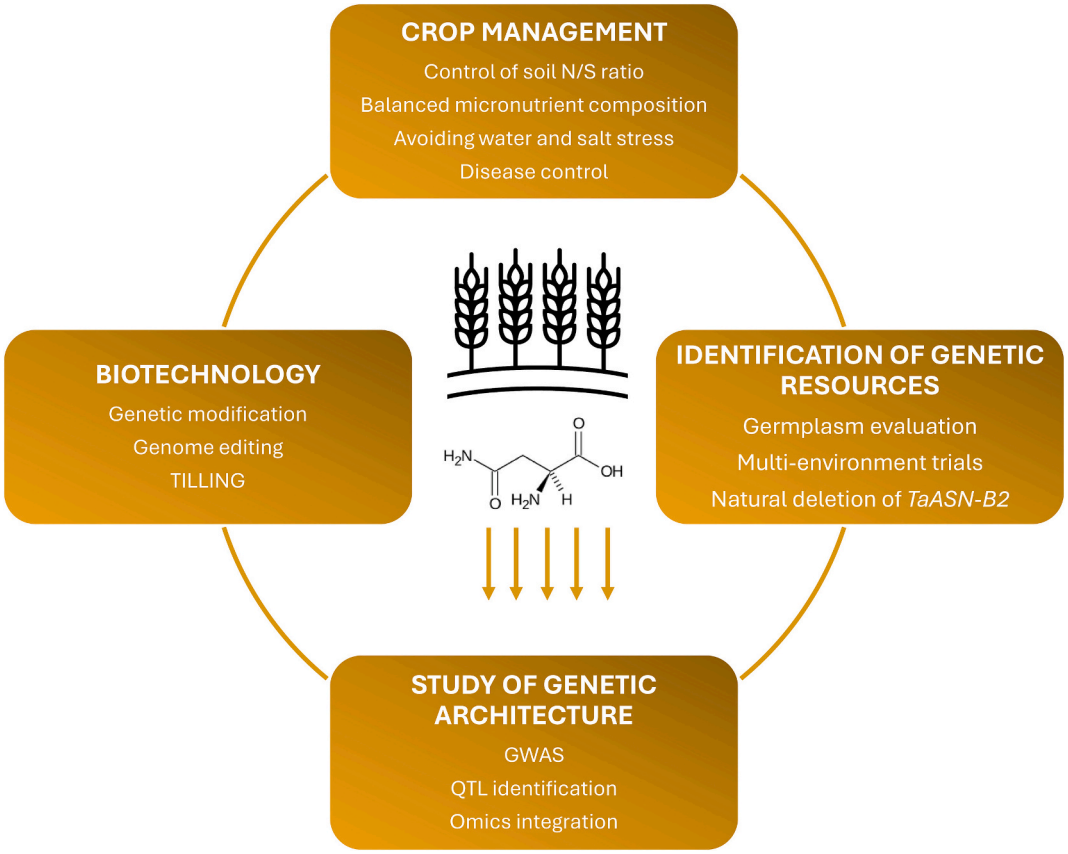

Figure 1. Graphical summary of the strategies adopted to obtain low free asparagine levels in cereal grain.

2. Field factors influencing free asparagine content

2.1. Asparagine metabolism and interaction with mineral nutrition

Asparagine functions as a major N storage and transport compound in plants, largely due to its high nitrogen-to-carbon ratio (Gaufichon et al., 2010). Free asparagine mobilizes N from source to sink tissues during germination, vegetative growth, senescence, and seed filling, and plays a critical role in ammonia detoxification. This detoxification becomes particularly important when N is abundant, such as under high N fertilizer inputs, or during biotic and abiotic stresses that cause ammonia accumulation in plant tissues (Zheng et al., 2018). During senescence or stress induced by pathogens and insects, N remobilization increases ammonia levels. Abiotic stressors can also trigger energy stress and proteolysis, leading to reduced cellular hexose concentrations and increased ammonia, which in turn promotes asparagine synthesis (Beato et al., 2014; Hildebrandt et al., 2015). Asparagine synthetase and asparaginase are the main enzymes regulating this process: the former catalyses the ATP-dependent conversion of aspartate to asparagine, while the latter hydrolyses asparagine to aspartate and ammonia (Oddy, Raffan, Wilkinson, Elmore and Halford, 2022). Asparaginase activity significantly increases as free asparagine concentration increases (Sodek et al., 1980), while it is strongly downregulated by glutamine concentration (Tonin & Sodek, 1990). The expression of these two enzymes is tightly regulated by environmental conditions: asparagine synthetase is typically upregulated by high N, while asparaginase activity is downregulated. Because of its sensitivity to both N supply and stress, free asparagine accumulation represents a critical indicator of N metabolism efficiency and the crop's response to soil and fertilization management. Table 1 summarizes the nutritional, environmental, biotic, and agronomic factors influencing free asparagine accumulation in plants, that are described in the following sections.

2.1.1. Macronutrients and free asparagine metabolism

Macronutrients are required in relatively large amounts and have a direct impact on plant growth, primary metabolism, and grain composition. N, phosphorus (P), and potassium (K) are particularly important for amino acid biosynthesis, protein formation, energy metabolism, and enzyme activation.

Most soil N (≈95–99%) occurs in organic forms that become available only after mineralization, while a small fraction is present as readily absorbable nitrate (NO_3^-) and ammonium (NH_4^+). In plants, N supports amino acid and protein synthesis and forms part of chlorophyll, influencing photosynthesis and carbon assimilation (Gupta, 2020). Imbalances between carbon and N metabolism can promote free asparagine accumulation (Gaufichon et al., 2010; Zheng et al., 2018). In condition of N surplus or deficiency, impaired protein synthesis redirects N into soluble nitrogenous compounds, such as free asparagine. Consequently, managing N availability is crucial for controlling free asparagine accumulation in cereals (Wilson et al., 2020).

P plays a pivotal role in plant metabolism by providing ATP and supporting key enzymatic activities involved in N assimilation. Adequate P availability during early plant development promotes root growth and branching, improving soil exploration and mitigating subsequent nutrient and water stress (Vance et al., 2003). P availability decreases under soil pH below 6.5 or above 7.5 and in the presence of high calcium (Ca), Mg, iron (Fe), or sodium (Na) levels. Excessive P fertilization can induce secondary deficiencies by reducing the uptake of Ca, Fe, K, copper (Cu), and zinc (Zn). Under P deficiency, an accumulation of free asparagine has been observed in soybean, tobacco, and ryegrass (Rufty et al., 1993, 1990). It is a result of restricted protein synthesis, caused by low ATP availability, and decreased carbon metabolism, which limit N incorporation into amino acids and favour its storage as asparagine.

K is another macronutrient that plays a key role in plant growth,

Table 1
Effects of nutrient availability, abiotic and biotic stresses, agronomic practices, and genetic factors on free asparagine metabolism in wheat. Arrows indicate the direction and magnitude of the effect on free asparagine: ↑ increase; ↓ decrease; ↑ strong increase; ↑↓ variable or context-dependent effect.

Category	Factor/stress		Effect on free asparagine	Physiological mechanism	Reference
Macronutrients availability	Nitrogen (N)	Excess	↑ Increase	Ammonium detoxification via asparagine synthesis	(Zheng et al., 2018)
		Adequate	↓ Decrease	Enhanced protein synthesis	(Soofizada et al., 2022)
		Deficiency	↑ Increase	Reduced protein synthesis	(Wilson et al., 2020)
	Phosphorus (P)	Excess	↑ Increase	Induced secondary deficiencies (Ca, Fe, Zn, Cu…)	(Murphy et al., 1981)
		Deficiency	↑ Increase	Reduced protein synthesis for low ATP availability	(Rufty et al., 1993)
	Potassium (K)	Excess	↑ Increase	Reduced uptake of N, B, Mg	(Lea & Fowden, 1975)
		Deficiency	↑ Increase	Reduced asparaginase activity	(Lea & Fowden, 1975)
Mesonutrients availability	Calcium (Ca)	Excess	↑ Increase	Reduced uptake of P, Zn, Mn, Fe	(Wdowiak et al., 2024)
		Deficiency	↑ Increase	Reduced membrane/cell wall stability	(Halford et al., 2015)
	Magnesium (Mg)	Excess	↑ Increase	Reduced K uptake	(Gransee & Führs, 2013)
		Deficiency	↑ Increase	Reduced photosynthesis	(Gaufichon et al., 2010)
	Sulphur (S)	Adequate	↓ Decrease	Enhanced N assimilation and protein synthesis	(Soofizada et al., 2022)
		Deficiency	↑↑ Strong increase	Enhanced asparagine synthetase activity	(Curtis et al., 2019)
Micronutrients availability	Zinc (Zn)	Excess	↓ Decrease	Inhibition of asparagine synthetase activity	(Lea & Fowden, 1975)
		Deficiency	↑↑ Strong increase	Impaired N assimilation and protein synthesis	(Possingham, 1956)
	Iron (Fe)	Deficiency	↑ Increase	Impaired nitrate reduction and protein synthesis	(Stewart & Larher, 1980)
	Molybdenum (Mo)	Deficiency	↓ Decrease	Reduced nitrate reductase activity	(Possingham, 1956)
	Selenium (Se)	Excess	↑ Increase	Possible S-starvation-like response	(Beshah et al., 2026)
		Deficiency	Decrease ↓	Enhanced oxidative stress mitigation	(Beshah et al., 2025)
Abiotic stresses	Salt (NaCl)	Excess	↑↑ Strong increase	Induced asparagine synthetase activity	(Herrera-Rodríguez, Pérez-Vicente and Maldonado, 2007)
	Salt + Nitrate (NO_3)	Excess	↑↑ Strong increase	Increased asparagine synthesis	(Carillo, Mastrolonardo, Nacca and Fuggi, 2005)
	Drought	Excess	↑ Increase	Increased asparagine synthesis for ammonium detoxification	(Yadav et al., 2019)
	Temperature	Excess	↑ Increase	Reduced protein synthesis	(Lea et al., 2007)
Biotic stresses	Fungal infection		↑ Increase	Increased proteolysis	(Curtis et al., 2016)
	Arbuscular mycorrhizal fungi		↑↓ Variable effect	Improved nutrient uptake; change in N availability	(Saia et al., 2015)
Agronomic practices	N fertilizer	Excess	↑ Increase	Increased asparagine synthesis for ammonium detoxification	(Fernández-Calvino et al., 2016)
	S fertilizer	Adequate	↓ Decrease	Avoided S deficiency	(Soofizada et al., 2022)
	High N:S ratio (>15:1)		↑ Increase	Induced S deficiency	(Liu et al., 2020)
	Organic farming		↑ Increase	-	(Stockmann et al., 2019)
	Fungicides application		↓ Decrease	Reduced fungal infection	(Martinek et al., 2009)
	Precision agriculture		↓ Decrease	Improved N use efficiency	(Fabbri et al., 2025)
Genotype-related	Stress-sensitive varieties		↑ Increase	Decreased N-use efficiency for protein synthesis	(Stockmann et al., 2019)
	Stress-tolerant varieties		↓ Decrease	Better regulation of N metabolism	(Stockmann et al., 2019)

enzyme activation, and N and asparagine metabolism. Excess soil concentrations of N, Na, P, or Mg can reduce K assimilation. Excessive K supply can in turn limit the uptake of N, boron (B), and Mg. K availability also influences asparagine metabolism, as K is required for the optimal activity of certain asparaginases. K deficiency has been associated with increased free asparagine accumulation in several plant species, possibly due to reduced asparaginase activity and imbalances in N assimilation (Lea & Fowden, 1975; Sodek et al., 1980). Appropriate K supply enhances crop resilience to stress and may stabilize asparagine metabolism. However, (Oddy et al., 2023) found that withholding P or K alone did not cause increases in free asparagine in wheat grain when S was applied.

2.1.2. *Mesonutrients*

Unlike macronutrients, which are required in large quantities, mesonutrients are needed at intermediate levels but remain essential for sustaining key metabolic and physiological processes in plants. Ca, Mg, and S contribute to enzyme activation, energy metabolism, nutrient transport, and cellular structure, thereby influencing amino acid biosynthesis, protein formation, and N assimilation.

Ca plays a key role in plant metabolism by stabilizing cellular membranes and reinforcing cell walls through interactions with pectins, thereby enhancing tolerance to biotic and abiotic stresses (Wdowiak et al., 2024). Because free asparagine accumulation in cereals is frequently associated with stress conditions and nutrient imbalances, sufficient Ca availability may indirectly help limit its synthesis and storage (Halford et al., 2015; Oddy et al., 2022). However, excessive Ca in the soil can interfere with the uptake of essential nutrients such as P, Zn, Mn, K, Mg, and Fe, potentially exacerbating stress responses and promoting further increases in free asparagine (Wdowiak et al., 2024). Therefore, maintaining balanced Ca levels is important to avoid physiological conditions that favour elevated free asparagine concentrations in grain.

Mg is essential as the central atom of chlorophyll, supporting

photosynthesis, and contributes to phosphorus mobility, carbohydrate metabolism, and enzyme activation. Crops respond positively to Mg fertilization when soil concentrations fall below 50 mg kg^{-1}. Excess Ca, K, and Na can reduce Mg uptake, while excess Mg can reduce K availability (Gransee & Führs, 2013). In agronomic practice, Mg/K ratios (expressed as cmol(+) kg^{-1}) between approximately 2.9 and 5 are generally considered adequate to ensure balanced cation nutrition. Differently, values below or above this range may increase the risk of Mg^{2+} or K^{+} deficiency, respectively. Mg availability also influences asparagine metabolism, as asparagine synthetase is Mg-dependent (Gaufichon et al., 2010; Lea & Fowden, 1975). High concentrations of K or Na inhibit asparagine synthetase activity, and Ca can nearly eliminate it, highlighting the importance of balanced cation nutrition (Lea & Fowden, 1975).

S plays a key role in N metabolism, enzyme activation, and protein synthesis (Kopriva et al., 2019) and is one of the most influential factors regulating free asparagine concentration in cereal grains (Curtis et al., 2019). S is essential for N metabolism, enzyme function, and protein synthesis. S deficiency enhances asparagine synthetase activity while suppressing asparaginase, thereby promoting free asparagine accumulation (Curtis et al., 2019). Conversely, adequate S supply consistently reduces free asparagine levels in wheat, with increasing S application rates leading to proportional decreases in free asparagine accumulation (Granvogl et al., 2007; Soofizada et al., 2022).

2.1.3. Micronutrients and enzymatic regulation

Mineral deficiencies, except for N and possibly molybdenum (Mo), generally increase the soluble N fraction in plants (Stewart & Larher, 1980), thereby favouring asparagine accumulation as part of N remobilization and stress response mechanisms. Micronutrients such as copper (Cu), iron (Fe), molybdenum (Mo), selenium (Se), and zinc (Zn) act as cofactors of enzymes involved in N assimilation and amino acid metabolism. Their deficiency can reduce protein synthesis efficiency and further promote the accumulation of free nitrogenous compounds, including asparagine.

Se contributes to antioxidant defence through incorporation into selenoproteins such as glutathione peroxidase, improving tolerance to abiotic stress (Hasanuzzaman et al., 2020; Yan et al., 2024). Foliar Se supplementation has been shown to reduce free asparagine in wheat grain (Beshah et al., 2025), potentially by interfering with its biosynthesis or promoting its turnover (Curtis et al., 2010; Curtis & Halford, 2016). Beshah et al. (2026) suggest that Se may impair Mo uptake and trigger S-starvation-like responses (White, 2018), thereby shifting metabolism toward S assimilation and lowering free asparagine accumulation (Soofizada et al., 2022; Wilson et al., 2020). Additionally, Se-enhanced antioxidant activity can mitigate stress-induced increases in free asparagine synthesis (Feng et al., 2013; Hasanuzzaman et al., 2020; Lea et al., 2007).

Zinc (Zn) plays important roles in enzyme activation, protein synthesis, and stress signalling. Its deficiency can severely disrupt N assimilation and amino acid metabolism, leading to substantial free asparagine accumulation. For example, Zn deficiency has been reported to increase free asparagine content in tomato shoots by up to 47-fold compared with controls (Possingham, 1956). Conversely, excess Zn can suppress asparagine synthetase activity, reducing asparagine biosynthesis (Possingham, 1956). Iron (Fe) deficiency similarly promotes free asparagine accumulation, likely due to impaired nitrate reduction and reduced incorporation of N into proteinaceous forms. In contrast, Mo deficiency may reduce free asparagine levels, as Mo is a cofactor of nitrate reductase. Reduced enzyme activity limits nitrate reduction to ammonium and diminishes the pool of assimilatory nitrogen available for asparagine synthesis (Possingham, 1956). These examples highlight how micronutrient imbalances modulate key steps of N metabolism and can markedly influence free asparagine accumulation in plant tissues.

Although data remain limited, micronutrient–asparagine interactions highlight the importance of comprehensive nutrient management approaches that go beyond macronutrients to ensure enzymatic homeostasis in asparagine metabolism.

2.2. Free asparagine accumulation under salt and water deficit stress

Salt stress profoundly alters N metabolism and promotes the accumulation of free amino acids in plants, including asparagine, glutamine, aspartate, glutamate, alanine, and proline. In *Arabidopsis thaliana*, NaCl treatment induces strong increases in free asparagine, suggesting a role for free asparagine in salinity tolerance (Maaroufi-Dguimi et al., 2011). This response is associated with the transcriptional induction of *ASN1*-type asparagine synthetase genes in both roots and leaves under 150 mM NaCl (Herrera-Rodríguez et al., 2007), and with enhanced asparagine synthetase activity, which elevates the free asparagine pool up to ~28-fold (~4.3% of total amino acids; Rashmi et al., 2019). Transient free asparagine accumulation has also been observed in sink tissues of *Coleus blumei*, peaking 5–15 days after salt exposure before returning to basal levels (Gilbert et al., 1998). Rapid free asparagine enrichment in roots, xylem sap, and leaves has been reported within two days of salinization in hydroponically-grown seedlings (Yamaya & Matsumoto, 1989), highlighting a role in short-term N redistribution.

Interaction between salinity and N supply modulates this response. Under saline conditions, free asparagine may increase up to seven-fold, particularly when high nitrate is available (*e.g.*, 100 mM KNO_3 combined with 100 mM NaCl; Carillo et al., 2005), implying that free asparagine serves both as a temporary N store and as an osmo-protectant under ion-induced osmotic imbalance. Species and genotype effects are evident: barley (*Hordeum vulgare*), for example, accumulates free asparagine proportionally to external NaCl concentration, unlike the more tolerant *Hordeum marinum* (Garthwaite et al., 2005), while salt-tolerant wheat germplasm exhibits stronger free asparagine accumulation than sensitive cultivars (Colmer et al., 1995). Collectively, these findings suggest that high free asparagine accumulation is often associated with improved salt adaptation via osmotic adjustment, N storage, and metabolic buffering.

Water deficit produces comparable metabolic signatures. In wheat, drought induces premature senescence, increased free asparagine content, and up-regulation of asparagine synthetase genes, particularly in less drought-tolerant varieties (Yadav et al., 2019). In rice, however, higher free asparagine levels negatively correlate with drought tolerance (Degenkolbe et al., 2013), illustrating species-specific metabolic strategies. Mechanistically, drought-driven senescence releases ammonium through proteolysis, and free asparagine synthesis detoxifies this ammonium while facilitating N remobilization to younger tissues. Under dehydration, free amino acids, including asparagine and proline, accumulate alongside morphological adaptations such as leaf folding and rolling, contributing to osmotic adjustment and cellular water retention (Kusaka et al., 2005). Together, these studies demonstrate that free asparagine metabolism is dynamically regulated by salt and water stress, serving integrative roles in osmoprotection, N storage, detoxification, and stress-related N transport.

2.3. Crop management strategies

The average free asparagine content in commonly cultivated cereal species ranged from 426 ± 144 mg kg^{-1} in common wheat to 1179 ± 359 mg kg^{-1} in rye in a study by Žilić et al. (2017). The cereals can be ranked according to increasing mean asparagine concentration as follows: common wheat < durum wheat < hull-less barley < dent maize < hull-less oat < rye (Stockmann et al., 2019; Szafrańska et al., 2025; Yiltirak et al., 2021; Žilić et al., 2017). Reduction of free asparagine in cereal grain can be achieved through various crop management strategies, although these have not been fully optimized (Oddy et al., 2022). The first initiative that was taken in this sense was introduced in the Commission Regulation (EU) 2017/2158, which included certain crop

management strategies as a part of the compulsory mitigation measures set out in the Regulation. These involved avoiding excessive N application while maintaining balanced S levels in the soil, as well as implementing good crop practices to prevent fungal infection. Current recommendations for reducing free asparagine in cereals, particularly in wheat, emphasise: i) the application of N fertiliser at the minimum levels to ensure that the optimum yield and required protein content is achieved, ii) the application of S fertiliser to ensure that S deficiency is avoided, and iii) the use of fungicides to control disease (FoodDrinkEurope, 2019). It is important to note that these recommendations do not fully address whether these practices are environmentally or economically sustainable, and their overall sustainability for farmers remains context dependent.

The effects of N and S fertilization on cereal grains are clear and well-established: high N fertilization promotes asparagine synthesis as plants detoxify excess ammonia by incorporating it into asparagine (Fernández-Calvino et al., 2016), leading to an increase in the concentration of free asparagine in the grain (Claus et al., 2006; Martinek et al., 2009; Granvogl et al., 2007; Curtis et al., 2016; Curtis, Bo, Tucker and Halford, 2018; Wilson et al., 2020; Szafrańska et al., 2024). S fertilization has the opposite effect; indeed, its deficiency leads to a substantial increase in free asparagine content in the grain, especially in wheat and barley (Granvogl et al., 2007; Curtis, Bo, Tucker and Halford, 2018). In rye, the effect of S deficiency under field conditions appears less pronounced (Postles et al., 2013), possibly due to greater efficiency of rye in S uptake and its different metabolic response compared to wheat and barley. Consequently, optimizing appropriate amounts of N and S fertilizers, as well as the N:S ratio, is essential to support plant growth and protein synthesis while minimizing acrylamide-forming potential. N application should be synchronized with crop demand through split applications, site-specific nutrient management, and precision fertilization tools. These approaches, including vegetation index-based canopy sensing and variable-rate fertilization, allow farmers to match N availability to crop uptake (Fabbri et al., 2025), minimizing surpluses that drive asparagine synthesis (Halford et al., 2022; Wilson et al., 2020). Field trials demonstrated that applying N and S at a 10:1 kg ha^{-1} ratio was sufficient to prevent large increases in free asparagine concentration in wheat grain (Oddy et al., 2023). Similarly, physiological studies in maize indicated that N:S ratios within the 10–15:1 range optimize amino acid balance, N assimilation, and redox status (Liu et al., 2020; Zhao et al., 1999).

Environmental factors also influence free asparagine accumulation. As previously stated, the asparagine content in grain is influenced by various abiotic and biotic factors, including drought, soil salinity, pathogen attack, toxicity, solar radiation, and nutrient deficiencies. These stress conditions trigger asparagine synthesis as a biological response to the restriction of protein formation (Lea et al., 2007; Navrotskyi et al., 2018). Biotic stresses have been shown to play an important role in free asparagine accumulation in wheat grain and an efficient fungicide treatment may decrease asparagine concentration (Lea et al., 2007; Martinek et al., 2009). Consistently, a correlation between the onset of serious fungal diseases and the increase in free asparagine concentration in wheat kernel was demonstrated, with the response varying among different wheat varieties (Curtis et al., 2016). Therefore, proper disease control *via* fungicide application is a primary crop management measure for mitigating acrylamide formation in cereal products. Arbuscular mycorrhizal fungi (AMF) inoculation may also be used to prevent fungi infection, although this practice may have side effects on asparagine accumulation due to higher supply of N and other nutrients (Saia et al., 2015; Whiteside et al., 2012).

Another factor that should be taken into consideration is the use of different agricultural practices between organic and conventional farming systems and their influence on the accumulation of free asparagine content. While most research has focused on conventional systems, studies on organic farming have produced inconsistent results. Springer et al. (2003) found higher free asparagine content in organically-produced rye, for example, whereas Stockmann et al. (2019) observed significantly lower levels of this amino acid in wheat cultivated under organic conditions compared with conventional systems. Moreover, the reduction in free asparagine content in spelt and rye was strongly influenced by the crop year (Stockmann et al., 2019).

3. Identification and creation of varieties of cereals with low free asparagine levels

Free asparagine concentration is the determining factor for acrylamide formation in cereal products. Hence, while there has been much attention paid to the development of processing strategies to reduce acrylamide formation, the provision of cereal varieties with consistently low concentrations of free asparagine in the grain would enable food businesses to comply with evolving regulations on acrylamide without costly changes to production lines or reduction in product quality. The use of these varieties could also have a significant impact on dietary acrylamide intake for consumers. Free asparagine content is influenced by genotype, environmental conditions, agronomic practices, and their interactions, making it a complex trait that poses challenges for prediction and improvement through conventional selection methods. Strategies aimed at lowering free asparagine levels and thereby reducing acrylamide formation in cereal-based products require an integrated approach combining varietal selection, molecular breeding, -omic technologies and biotechnologies. The following sections describe the current knowledge related to the identification of low asparagine genotypes in breeding programmes, the development of new, low asparagine varieties by conventional breeding methods, and the potential for biotechnology to be used to bring about a step reduction in free asparagine concentrations to below the range seen in current varieties.

3.1. *Assessing diversity of asparagine levels*

Several studies have reported data on the differing propensities of cereal genotypes to accumulate free asparagine in the grain ((Žilić et al., 2017; Curtis et al., 2010; Postles et al., 2013; Corol et al., 2016). Most of the attention has been focused on wheat, due to its agronomic and commercial importance (Curtis, Bo, Tucker and Halford, 2018; Navrotskyi et al., 2018; Wilson et al., 2020; Xie et al., 2021; Oddy et al., 2023; Tafuri et al., 2023; Szafrańska et al., 2024; Spanic et al., 2025; Tafuri et al., 2025). Some studies highlighted that certain genotypes maintain low and stable levels of free asparagine in the grain. For instance, eight bread wheat varieties with low free asparagine concentration in the grain, regardless of crop management and growing year, were identified by Curtis and coworkers (Curtis, Bo, Tucker and Halford, 2018). More recently, further bread wheat cultivars, relevant for the Italian market, have been found to exhibit relatively stable free asparagine content across different years and locations, *i.e.*, three Italian locations over two crop years (Tafuri et al., 2023). Interestingly, this study took advantage of the Italian experimental trial network, which evaluates wheat varieties across multiple locations to assess qualitative performance: the 54 analysed bread wheat cultivars were included in the Italian "National Network of Common Wheat Variety Comparison" (In Italian: "Rete Nazionale di Confronto Varietale del Frumento Tenero") coordinated by the Council for Agricultural Research and Economics (CREA).

The cultivars identified in these studies represent valuable donor lines for breeding programs targeting acrylamide mitigation. Despite the strong influence of environmental factors, these studies have pointed out an important role of genetics in determining free asparagine concentration. They also have suggested that the selection of low-asparagine varieties could be a successful approach in breeding to reduce acrylamide-forming potential. Importantly, lowering free asparagine levels does not negatively affect end-use quality or technological performance (Oddy et al., 2022; Tafuri et al., 2023), supporting the feasibility of breeding low-asparagine wheat varieties without compromising

product quality.

3.2. Towards low-asparagine cereals: genetic determinants and environmental interactions

Few genetic studies have been carried out to dissect the genetic bases of free asparagine accumulation in cereal grain so far, and most of them related to wheat. Genome wide association studies (GWAS) conducted on bread and durum wheat have identified 70 and 6 marker-trait associations (MTAs), respectively, linked to free asparagine (Table 2).

The first GWAS related to free asparagine accumulation in wheat was performed on 92 bread wheat genotypes used for breeding in Australia (Emebiri, 2014). This study identified a region on chromosome 5A defined by nine SNPs. However, because the analysis was carried out under greenhouse conditions, the findings may not be fully applicable to wheat grown in the field. A further GWAS was conducted on 149 European bread wheat varieties grown under field conditions (Rapp et al., 2018). Eight Quantitative Trait Loci (QTL) on chromosomes 1A, 4A, 7A, 1B, 6B, 7B, 2D and 6D associated with free asparagine accumulation were identified, all showing medium-high heritability. The genetic architecture of free amino acid composition in wheat grain was also studied using a mapping population of 171 doubled haploid lines by Oddy and colleagues (Oddy et al., 2023). This study identified a QTL on chromosome 4B that was significantly associated to free asparagine accumulation. More recently, both single-locus and multi-locus association studies were performed on 417 bread wheat accessions, identifying nine QTL for free asparagine content located on eight chromosomes (Lavoignat et al., 2024). Similarly, Rączka et al. (2025) detected 43 MTAs on fifteen chromosomes in 174 elite winter wheat lines, further confirming the polygenic nature of this trait. In durum wheat, Tafuri et al. (2025) performed a GWAS on 201 genotypes, principally landraces, and identified six significant SNPs on chromosomes 6A, 7A, 2B, 4B, and 7B using data collected over three years of field trials.

Multi-environment GWAS analyses have revealed considerable instability in MTAs, with many loci exhibiting environment-specific effects. For example, Lavoignat et al. (2024) observed average increases in free asparagine content of 4% under high N supply and 3% under water-deficit conditions. To date, no major-effect QTL have been identified, with individual markers typically explaining only 6.3% to 12.2% of the phenotypic variance (Rączka et al., 2025). These findings underscore the complex genetic architecture of the trait, which is governed by multiple loci with moderate additive effects. Strong G×E interactions, along with potential pleiotropic effects on agronomic traits, such as yield and grain quality, further complicate breeding strategies. These interactions highlight the need for multi-environment trials and integrative approaches to select genotypes with low free asparagine levels without compromising other key traits. Nevertheless, the heritability of free

Table 2
Number and position of peak markers of the MTAs and QTL identified for free asparagine content in Triticum aestivum and turgidum.

Species	Reference	Reference Genome	N. of peak markers	Chr	Chromosomic region (Mbp)
T. aestivum ssp. *aestivum*	(Emebiri, 2014)	IWGSCv1.0	9	5A	585.1 - 588.7
	(Rapp et al., 2018)	IWGSCv1.0	1	1A	307.4
			1	4A	743.8
			1	7A	54.9
			1	1B	40.8
			1	6B	164
			1	7B	750.6
			1	2D	19.8
			1	6D	467
	(Oddy et al., 2023)	IWGSCv1.0	1	4B	601
	(Lavoignat et al., 2024)	IWGSCv2.1	1	1A	464.5
			1	1B	515.2
			1	2B	794.7
			1	2D	610.4
			1	3B	781.3
			1	5B	295.1
			1	7A	706.7
			2	7B	176.2; 177.8
	(Rączka et al., 2025)	IWGSCv2.1	4	1B	468.9 - 495.3
			3	1D	20.5; 368.0; 423.0
			3	2B	31.5; 111.5; 516.7
			2	2D	35.8; 496.8
			1	3A	0.9
			3	3B	2.5; 256.1; 837.2
			1	3D	40.6
			1	4A	698.2
			2	5B	634.2; 697.9
			1	5D	122.2
			7	6B	18.8; 245.3; 313.1; 323.2; 356.0; 375.6; 708.3
			2	6D	181.1; 418.4
			8	7A	25.8; 413.0; 477.5; 549.9; 622.0; 638.9; 656.7; 702.8
			1	7B	756.0
			4	7D	15.4; 77.7; 114.5; 633.0
T. turgidum ssp. *durum*	(Tafuri et al., 2025)	Svevo v1	1	6A	132.5
			1	7A	688.4
			1	2B	405.2
			1	4B	609.4
			2	7B	545.2; 697.6

asparagine content is medium to high (Lavoignat et al., 2024; Rączka et al., 2025; Tafuri et al., 2025), supporting the feasibility of genetic selection, especially when supported by stability analyses and data from diverse growing environments.

Integrative metabolomic analyses have highlighted the involvement of pathways related to amino acid metabolism, aromatic compound biosynthesis, and redox balance in regulating free asparagine accumulation. Tafuri et al. (2025) combined GWAS and metabolomics to identify 40 metabolites correlated with free asparagine variation, demonstrating that such integrative approaches are essential to uncover novel regulatory components and deepening our understanding of the metabolic networks involved. Thanks to high-quality cereal reference genomes International Wheat Genome Sequencing Consortium (IWGSC), 2018; Maccaferri et al., 2019), MTAs and QTL can now be linked to candidate genes potentially involved in free asparagine regulation (Table 3).

The integration of GWAS and metabolomics identified some enzymes associated with N metabolism, oxidative stress, and aromatic compound biosynthesis (*i.e.*, L-lactate dehydrogenases, polyamine oxidases, and anthranilate phosphoribosyl transferase) as putative candidate genes (Tafuri et al., 2025). Similarly, the gene *TraesCS1B03G0736700*, encoding an H-subunit of mitochondrial NAD(P)H dehydrogenase that is involved in N metabolism and redox balance, has been proposed as a candidate for free asparagine regulation (Rączka et al., 2025).

Recently, a network describing asparagine metabolism was built using information available in literature and in public databases (Curtis, Powers, Wang and Halford, 2018). This analysis highlighted the complexity of this network, which includes 212 nodes (genes, enzymes, or molecules) and 246 edges (reactions between nodes). The main enzymes involved in asparagine metabolism are: asparagine synthetase, glutamine synthetase, glutamate dehydrogenase, ferredoxin-dependent glutamate synthase, NADH-dependent glutamate synthase, aspartate amino transferase, and glutamate decarboxylase. In addition, specific kinases and transcription factors were identified as regulators of gene expression within this network (Curtis, Powers, Wang and Halford, 2018). Asparagine metabolism is highly branched and dependent on several factors, and the precise mechanisms regulating its accumulation in grains remain unclear. Notably, many QTL associated to free asparagine levels do not co-localize with known asparagine metabolism genes. This suggests that additional regulatory elements or less obvious pathways, such as transport, degradation, secondary metabolism, or epigenetic control, may contribute to this metabolic network. These observations underscore the need for functionally validating candidate genes *via* expression profiling, mutagenesis, or genome editing.

3.3. The potential for biotechnology to reduce the free asparagine content of cereal grains

By biotechnology we mean genetic modification (GM) and genome editing (GE), but we are not aware of GM approaches being used to reduce the free asparagine content of cereal grains. In contrast, GM has already been used successfully in the USA to produce low acrylamide potato varieties. These varieties, Innate® (approved in 2015) and Innate® Generation 2 (approved in 2017) were developed by the J.R. Simplot Company ((USDA-APHIS, 2013; USDA-APHIS, 2014; Chawla et al., 2012) and exhibit strong reductions in acrylamide formation through targeted downregulation of the *StASN1* gene, encoding asparagine synthetase, by RNA interference (RNAi), specifically in tubers. Two genes encoding enzymes of starch breakdown, namely phosphorylase L (*PhL*) and starch-associated R1 (*R1*), and a gene encoding polyphenol oxidase (*PPO5*), an enzyme involved in bruising, are also down-regulated by RNAi. Innate 2 also incorporates Blight resistance (*Rpi-vnt1R* gene) and reduced vacuolar invertase (*VInv* gene) to reduce cold sweetening during storage. Field evaluations under U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) oversight reported up to 70% reductions in acrylamide formation during the preparation of French fries, with no detrimental impact on yield or quality. Despite their commercial success in the United States and Canada, as well as their clear food safety benefit, these GM potatoes remain unavailable to European consumers due to the EU's risk assessment and approval process for GM crops and their products.

Regarding the reduction of free asparagine content in cereal grains, the emphasis has shifted to the newer technique of GE, partly because of the relative simplicity of editing based on Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associate nucleases (Cas), in particular CRISPR/Cas9. Bread wheat (*Triticum aestivum*) has five asparagine synthetase genes in each of its three genomes, A, B and D (Xu et al., 2018). These are *TaASN1*, *TaASN2*, *TaASN3.1*, *TaASN3.2* and *TaASN4*. *TaASN2*, highly expressed in embryo and endosperm (Curtis et al., 2019; Xu et al., 2018), is absent on the B genome (*TaASN-B2*) in some cultivars due to a natural deletion, correlating with reduced grain asparagine (Oddy et al., 2021). This remains the only QTL validated across multiple environments to date (see previous section).

The development of lines in which *TaASN2* was knocked out using CRISPR/Cas9 was reported in 2021 (Raffan et al., 2021) and a field trial of an A genome null for *TaASN2*, and a total null for *TaASN2* was carried out in 2021 to 2022 (Kaur et al., 2024; Raffan et al., 2023). Also included were AB genome nulls derived from a selected line of a mutant population of wheat cv. Cadenza seeds that was produced by chemical

Table 3
Candidate genes, and associated markers, potentially involved in free asparagine regulation from literature.

Species	Candidate gene	Gene name	Metabolic pathway	Chr	Associated QTL	Peak marker position (Mbp)	Reference
T. aestivum	*TraesCS3A02G077100; TraesCS3D02G077300*	asparagine synthetase 2 (*TaASN2*)	asparagine synthesis in seed	3A, 3D	-	-	(Raffan et al., 2021)
	-	asparagine synthetase 2B (*TaASN-B2*)	asparagine synthesis in seed	3B	-	-	(Oddy et al., 2021)
	TraesCS1B03G0736700	H-subunit of mitochondrial NAD(P)H dehydrogenase	mitochondrial electron transport chain	1B	QGfac.rut.1B.1	468.9	(Rączka et al., 2025)
T. turgidum ssp. durum	*TRITD4Bv1G182260*	anthranilate phosphoribosyl transferase (*PAT1*)	tryptophan biosynthesis pathway	4B	GENE-4008_418	609.4	(Tafuri et al., 2025)
	TRITD7Bv1G172590	L-lactate dehydrogenase (*LDH*)	glucose metabolism / asparaginase II pathway	7B	BS00108573_51	545.2	(Tafuri et al., 2025)
	TRITD7Bv1G172680	L-lactate dehydrogenase (*LDH*)	glucose metabolism / asparaginase II pathway	7B	BS00108573_51	545.2	(Tafuri et al., 2025)
	TRITD7Bv1G225350	polyamine oxidase (*PAO*)	polyamine metabolism /asparagine-methionine bicycle	7B	BS00062611_51	697.6	(Tafuri et al., 2025)

mutagenesis through ethyl methanesulphonate treatment (Rakszegi et al., 2010), referred to as TILLING lines. The mutated *TaASN2-A2* gene had been backcrossed into the cv. Claire background, which already lacks a B genome *TaASN2* gene (Oddy et al., 2021). The CRISPR lines showed a significant reduction of free asparagine concentration in the grain compared with the Cadenza control, by just over 50% in the total nulls and 14% in the A genome null (Kaur et al., 2026; Raffan et al., 2023). The TILLING lines showed more variable responses when compared to Claire; however, when compared with a TILLING control that had come through the mutagenesis process but did not carry mutations in *TaASN2*, they did show significant reductions of 20-40%, comparable with the 28% reduction that was previously evaluated in A genome TILLING nulls in the Cadenza background and the tetraploid Kronos background (Alarcón-Reverte et al., 2022). Notably, the large reductions in free asparagine concentration in the CRISPR lines was achieved without significant effects on yield (Raffan et al., 2023), although there was evidence of a yield drag in TILLING lines, possibly due to the genetic baggage of additional random mutations that they will inevitably carry.

A second *TaASN2* null line was found to have additional edits in the *TaASN1* genes (Kaur et al., 2024). This line and a total *TaASN2* TILLING line are still being evaluated in the field but are likely to have even greater reductions in free asparagine concentration.

These results show that step reductions in the free asparagine concentration of wheat grain are possible using GE or chemical mutagenesis (TILLING), suggesting that these strategies could offer a critical path forward to the development of crop varieties with inherently low acrylamide-forming potential (Figure 2). In this context, the identification of additional genetic determinants controlling the levels of free asparagine and possibly reducing sugars has become an important target for crop geneticists. However, species-specific considerations are critical because the five-gene asparagine synthetase gene family that lends itself so well to genetic intervention is unique to the *Triticeae* (Raffan & Halford, 2021). Rice, for example, possesses only two asparagine synthetase genes, *OsASN1* and *OsASN2* (orthologous to wheat *TaASN4* and *TaASN3,* respectively; Raffan & Halford, 2021). CRISPR/Cas9-mediated editing of these genes results in drastic reductions in free asparagine, with levels dropping to approximately one-sixth of wild-type. However, disruption of *OsASN1*, either through T-DNA insertion or CRISPR/Cas9 mutagenesis, leads to severe developmental defects, including decreased height, biomass, and tiller number, despite unchanged total N uptake (Luo et al., 2019). Such findings indicate that species with smaller asparagine synthetase gene families may be less amenable to genetic interventions.

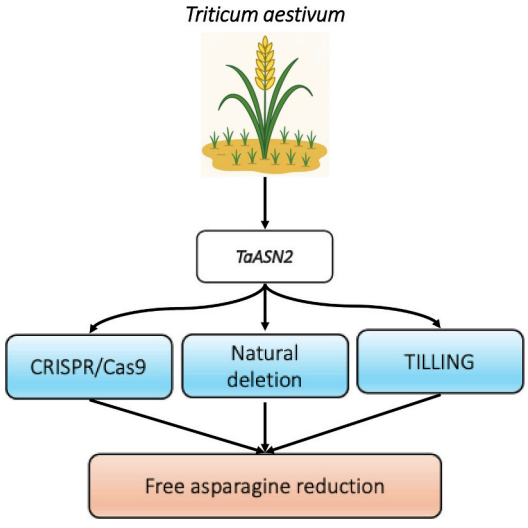

Figure 2. Biotechnological approaches to obtain wheat genotypes with low levels of free asparagine in grain.

3.3.1. *European legislation for plants subjected to genome editing*

To our knowledge, the successful biotechnological strategy used by Halford's research group for lowering free asparagine in cereals is the only one on the European scientific horizon. One of the main factors that has halted progress is the restrictive European regulatory framework, which classifies GE alongside GM crops (Halford, 2019). In the United Kingdom, the Genetic Technologies (Precision Breeding) Act (2023) was introduced to create a more enabling environment for innovation using GE, and applications for marketing of GE crops have been considered from November 2025. However, the Act has only been adopted in England and the fact that the other parts of the UK are dragging their feet means that commercialisation of GE commodity crops such as wheat will remain difficult.

Regarding EU regulation, currently all plants obtained with GE are subjected to the same rules as genetically modified organisms (GMOs), contained in Directive 2001/18/EC and Food and Feed Regulations (EC) No 1829/2003 and (EU) No 503/2013. In 2022, EFSA published two documents ("Updated scientific opinion on plants developed through cisgenesis and intragenesis" https://www.efsa.europa.eu/en/efsajournal/pub/7621, and "Criteria for risk assessment of plants produced by targeted mutagenesis, cisgenesis and intragenesis" https://www.efsa.europa.eu/en/efsajournal/pub/7618) containing important contributions to the possible revision of the regulations to allow GE plants to undergo a less onerous risk assessment process than that applied to GM crops. After that, and also after consultations with stakeholders and the general public, on July 5th 2023 the EC has proposed to the EU Parliament and the EU Council a specific "Regulation on plants obtained by certain new genomic techniques and their food and feed" (COM(2023) 411), which was considered a better option than proposing changes on Directive 2001/18/EC. In the proposal, plants produced with new genomic techniques (defined as including GE and cisgenesis) are divided into two different categories, depending on the nature of the genetic mutations they carry: i) plants with mutations that could also have been obtained naturally or by conventional breeding techniques, named NGT Category 1, are exempted from the requirements of the GMO legislation and are subject to the provisions applicable to conventional plants; ii) the other plants produced with the same techniques but with more complex modifications (NGT Category 2) will continue to be treated as GMOs, but with some regulatory incentives in order to adapt the procedures laid down in Directive 2001/18/EC to the specific nature of the plants and the differing levels of risk that they may pose. On February 7th, 2024, the EU Parliament approved the EC proposal with some modifications. On March 7th, 2025, the EU Council approved a text with its modifications of the proposal, thus starting the process of discussion between the three EU Institutions (trilogue) to reach an agreement on the final text to be adopted as an EU legislation. On April 2026, the regulation on plants obtained by NGT and their products was finally adopted. Although progress on bringing forward legislation has been slow, the recent approval of the new regulation gives a novel perspective to the use of genome edited plants in agriculture.

4. Developing fast techniques for screening free asparagine level in cereals

Monitoring free asparagine content is essential for both the selection of raw materials and the analysis of raw food ingredients. For this reason, the development of new and improved methods for this measurement is of great interest for all the actors of the supply chain, form breeders to food technologists.

The analytical techniques mostly used for the analysis of free asparagine in biological materials are based on chromatography and mass spectrometry (HPLC, UHPLC, GC, GC-MS or LC-MS, LC–MS–MS) and are performed on partially purified and derivatised amino acid fractions (Palazoğlu et al., 2015; Žilić et al., 2017; Curtis, Bo, Tucker and

Halford, 2018; Žilić et al., 2020). These conventional chromatographic methods offer high sensitivity but require a long and tedious multi-step sample preparation. Indeed, these techniques are complex, time consuming and expensive, requiring dedicated and expensive devices such as amino acid analysers or special detectors. Optimization of extraction conditions, removal of proteins and other components from the analysed matrix, as well as required derivatization procedures, further complicate this process. Some procedures for these techniques have been updated to decrease the time for the analysis. Phenomenex, for example, has developed the EZ:faast® Amino Acid Analysis Kit (Phenomenex, Inc., Torrance, CA), which requires just 15 min for sample preparation and analysis, for the measurement of free asparagine in potato samples (Farkas & Toulouee, 2003). This procedure requires a simple solid phase extraction (SPE) step, followed by a fast derivatization reaction and GC (FID/NPD) analysis. Alternative kits with simplified procedures are available for either GC/MS or LC/MS analysis (Farkas & Toulouee, 2003).

Another technique that can be adopted for the measurement of free asparagine is ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR). Corol et al. (2016) described the development and application of a rapid ^{1}H-NMR-based analysis of cereal flour for free asparagine quantification. Tafuri et al. (2025) confirmed the reliability of this analytical method. ^{1}H-NMR is quantitative and does not rely on the use of calibration curves. Extraction of chemical compounds involves simple procedures, with no need for derivatisation. However, accurate quantitation of individual compounds in complex compound fingerprints strictly depends on the presence of molecule-specific resonances nonoverlapping with other signals in the spectra. According to the protocol described by Corol et al. (2016), the characteristic, geminally-coupled signals of the asparagine C-3 hydrogen atoms fall in a relatively clear area of the spectrum and thus are suitable for integration. The cleanest signal of the pair at δ2.95 (dd, J = 17 and 4 Hz) arising from 3-Hb was used by authors for integration and quantitation. The selected peaks were accomplished by comparison to the known concentration of the d4-TSP standard. It is then clear that the use of ^{1}H-NMR requires specific and expensive instruments, as well as high specialized personnel.

Breeders and food manufacturers need more straight-forward and faster methods to analyse raw materials. These methods should ensure that the measurement of free asparagine is easy, instantaneous and reliable. For this reason, in recent years activities have been focused on developing new user-friendly methods, such as enzymatic and spectrophotometric approaches, that would find application directly in breeding companies, food industries, raw material receiving points, warehouses, etc.

An interesting product is the enzymatic assay "L-Asparagine/L-Glutamine/Ammonia Assay Kit" (K -ASNAM Megazyme, Illinois, USA; https://www.megazyme.com/l-asparagine-l-glutamine-ammonia-rapid-assay-kit), which only needs a spectrophotometer (UV/VIS) to measure the absorbance of the sample solutions. This kit contains a coloured reagent required for spectrophotometric quantification at λ 340 nm (nicotinamide-adenine dinucleotide phosphate, NADPH), as well as three specific enzymes (glutaminase, glutamate dehydrogenase (GIDH) and asparaginase), and their appropriate buffers. This kit is suitable for the analysis of L-asparagine, L-glutamine and ammonia. The detection of these compounds takes place in a three-step reaction. Reaction 1: glutamine from the sample is converted to glutamate and ammonium ions. Reaction 2: both ammonium ions from the sample and from reaction 1 react with 2-oxoglutarate and NADP to form glutamate and $NADP^{+}$. Reaction 3: asparagine is hydrolysed to aspartate and ammonium ions and then the liberated ammonium ions enter into reaction 2, which leads to a fall in absorbance that is stoichiometric with the amount of asparagine (Figure 3).

This method has been adapted for wholemeal wheat flour, with quantification of free asparagine from the sample carried out after total deproteinization (Lecart et al., 2018). Xie et al. (2023) validated the enzymatic kit protocol on wholemeal flour through the comparison with

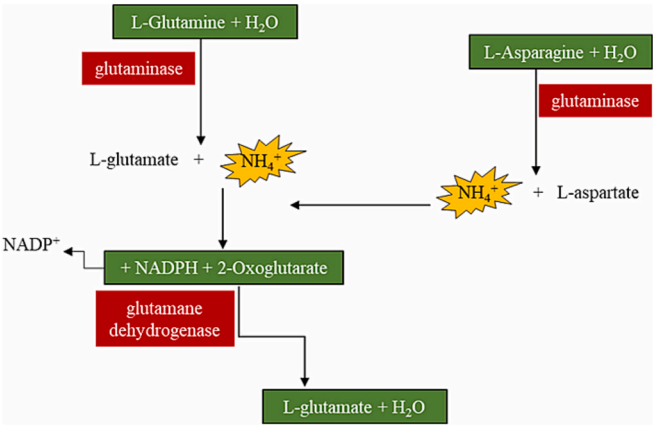

Figure 3. Reaction flow diagram according to the description of the method proposed by "L-Asparagine/L-Glutamine/Ammonia Assay Kit" manufacturer (Megazyme, 2018).

the two well-established analytical methods, Ultra High-Performance Liquid Chromatography (UHPLC) and ^{1}H-NMR. A strong correlation between the enzymatic test and UHPLC was obtained. In particular, the authors stated that the enzymatic test had consistent accuracy, excellent precision of 1.0 or less (CV%), good sensitivity (0.0389 AU L mg^{-1}) of free asparagine signal detection, as well as the lowest cost. Recently, the comparison of this enzymatic assay *vs* UHPLC for free asparagine determination in pea and lentil seeds was carried out by (Sá et al., 2025). The procedure set up by Lecart et al. (2018) has been used successfully in several studies on free asparagine accumulation in wheat grain (Covino et al., 2024; Guarino et al., 2026; Lavoignat et al., 2024; Tafuri et al., 2023; Tafuri et al., 2025). This method is simple and cheap, but the procedure (acid deproteinization and enzymatic assay) is more suitable for research activities than for use by seed or food companies.

More recently, Alhazzani et al. (2025) presented a novel and selective method for the determination of free asparagine on potato, based on carbon dots (CDs): nanoscale particles known for their excellent photoluminescent properties. Upon enzymatic hydrolysis of free asparagine by L-asparaginase, liberated ammonia induced a pH increase in the reaction medium. This pH shift enhanced the fluorescence of blue-emitting CDs (B-CDs) while simultaneously decreasing that of orange-emitting CDs (O-CDs), enabling sensitive and selective free asparagine quantification. This method displayed excellent sensitivity (LOD = 0.31 μM) with low relative standard deviation, confirming its accuracy and precision. Fluorescence emission spectra were recorded using a spectrofluorometer. The use of CDs offers several advantages, such as low toxicity, low environmental impact, and versatility, making this method suitable for a wide range of applications (Alhazzani et al., 2024).

5. Perspectives and recommendations on future research to lower free asparagine content in cereal grain

5.1. Soil and crop management

Future research on the effect of crop management strategies on free asparagine accumulation should address several key aspects. A holistic approach is required to determine the optimal levels and ratios of N and S fertilization, as well as the influence of other agronomic practices, to develop effective and sustainable strategies for mitigating acrylamide formation in food. Integrated soil fertility management practices, such as the use of organic amendments and cover crops, can further improve N retention, enhance N use efficiency, and reduce leaching losses (Drinkwater & Snapp, 2007), thereby indirectly supporting the control of free asparagine accumulation. In addition, the use of precision agriculture could improve N-use efficiency through N application

synchronized with crop demand.

A comprehensive and balanced approach to soil management and fertilization is essential to control free asparagine accumulation in cereal grains. Integrating precision nutrient management, regular soil testing, S supplementation, and micronutrient balancing can effectively reduce free asparagine levels while maintaining yield and grain quality. Looking ahead, coupling these agronomic practices with innovations in soil biology and digital agriculture offers a sustainable pathway to reduce acrylamide risk in cereal-based foods, improve crop N efficiency, and enhance resilience in future agricultural systems. Research should also address the effects of agronomic practices beyond fertilization, such as fungicide application, leaf area duration, and senescence control to evaluate their potential for reducing free asparagine and mitigating acrylamide formation. In addition, it is necessary to better understand the impact of abiotic stresses, particularly drought and salt stress, on free asparagine accumulation in cereals. Also, the use of AMF to counteract environmental stresses and simultaneously decrease free asparagine content needs to be further investigated.

5.2. Identification of genetic resources for breeding

Breeding low-asparagine wheat varieties without compromising product quality is a possible target, as shown in Section 3. To achieve this goal, diversity assessment through germplasm screening for the identification of genetic resources with low free asparagine content is essential. These sources should also perform well in other key traits including grain yield, disease resistance and baking quality if farmers and food businesses are to use them. The methodology to accomplish this objective includes: i) characterization of genotypes for free asparagine content, using, for instance, germplasm collections; ii) selection of varieties that exhibit the widest possible range of free asparagine content; iii) establishing field trials with the selected varieties at different locations to evaluate the stability of the trait across environments.

Most studies reported in the literature evaluated a limited number of varieties. A promising strategy to assess diversity for this trait is the investigation of asparagine level across a broad range of European elite varieties, as well as non-European varieties (global collections), landraces and representative sets of cereal species that have been less studied to date, including durum wheat, spelt, rye, emmer and einkorn. The screening of this large diversity panel should be conducted across multiple environments and under different farming practices, with particular focus on mineral fertilization and fungicide treatments. This will allow the identification of genotypes with consistently stable asparagine content, which can then be prioritized for use in pre-breeding and breeding. For this kind of screening, it would be desirable to leverage varieties that are already included in national experimental trial networks in some European countries (*e.g.,* Italy, Germany and UK), as reported in the study by Tafuri et al. (2023). These networks annually evaluate varieties across multiple locations, representing an important tool for orienting breeders in the current varietal landscape. Coordination of this network is usually managed by a research organization with the support of several collaborators belonging to public and private institutions. Testing the grain from these varieties for free asparagine content by both official governmental bodies and private companies, giving multiple replicates across diverse environments, would provide an efficient starting point for identifying the best varieties currently available on the market.

Another important aspect is the coordination of this kind of experiment among different countries, which can further enhance the assessment of genotype stability under diverse environmental conditions. Information on which European varieties contain low concentrations of free asparagine would be useful as a recommendation for farmers regarding the appropriate varietal choice to produce purpose-built raw materials for the food industry. Furthermore, based on these data, authorized institutions could develop a database to set targets for the reduction of free asparagine levels in cereal grains. Including free asparagine levels in standard cultivar information, potentially as part of regulatory criteria, should be evaluated. From both agronomic and breeding perspectives, the ambition to assess the diversity of asparagine levels in cereal cultivars and the identification of genetic resources for breeding purposes should be specified within guidelines.

5.3. Integration of genetics and -omic technologies

The integration of genomics and quantitative genetics plays a pivotal role in dissecting the genetic architecture underlying free asparagine accumulation and, in turn, the selection of cereal varieties with low and stable levels across environments. We discussed in Section 3.2 how GWAS can help to identify genomic regions and candidate genes involved in this trait. To exploit their potential in dissecting a complex trait like free asparagine accumulation, these studies should be designed using large, genetically diverse populations, incorporating environmental and agronomic data as covariates to increase statistical power and enable the detection of minor-effect QTL. Expanding genetic diversity through the exploration of wild relatives, landraces, and ancient subspecies is essential for identifying novel alleles associated with reduced free asparagine content. On the other hand, multi-parental populations such as Nested Association Mapping (NAM) and Multi-parent Advanced Generation Inter-Cross (MAGIC) provide valuable resources for dissecting complex traits due to their high recombination rates and broad genetic backgrounds (Kitony, 2023; Samantara et al., 2021).

The integrated use of –omic techniques represents a powerful strategy to dissect the genetic architecture underlying free asparagine metabolism. Genomics can identify chromosomic regions associated with the trait of interest, as well as putative candidate genes involved in free asparagine metabolism. Proteomics can provide insights into the protein interactions involved in asparagine metabolism, while combining genetic and proteomic data offers a more comprehensive understanding of the mechanisms controlling free asparagine accumulation. Metabolomics, in turn, provides a detailed profile of cereal grain composition, enabling the identification of molecules whose levels correlate with free asparagine accumulation.

The combination of these -omic datasets can uncover novel regulatory components of free asparagine metabolism in cereal grains. Exploiting this complex network will ultimately facilitate the identification of genes with a key role, thus taking advantage of the potential of genome editing on novel targets for lowering free asparagine accumulation.

Finally, genomic selection represents a promising breeding strategy for reducing free asparagine levels in the grain, particularly when low acrylamide-forming potential is incorporated as a selection criterion within genomic prediction models.

Such integrative approaches may accelerate genetic gain and facilitate the identification of lines that combine stable, low free asparagine content with strong agronomic performance across diverse environments.

6. Conclusions

Lowering free asparagine content in cereal grains implies the use of different and complementary actions that range from agronomic strategies to advanced breeding programs and the use of biotechnological approaches. Several studies have been reported in the last two decades about the environmental and agronomic factors that influence this trait, and the related information is increasing. Obtaining and sharing data is not just of value for farmers and food business operators, but also for important authorized institutions. This brings together plant breeders, the farming sector, food processors, toxicologists, public regulators, and consumer advocacy groups to establish research standards for free asparagine accumulation in plants. Sharing data could bring strong improvements in the management of free asparagine accumulation in

cereal grains:

i) gathered information may be of great importance for farmers, helping them to select appropriate cereal varieties and implement good agricultural practices to produce raw materials with low free asparagine content;

ii) enhancing farmers' awareness and adoption of best crop management practices through targeted education, training, and outreach initiatives remains crucial for minimising asparagine levels in cereal production systems;

iii) continuous improvements in agronomic practice are crucial to ensure regulatory compliance and food safety, and to reduce potential acrylamide-associated risks to consumers' health.

On the other hand, slower progress has been made in the creation of cereal varieties with low asparagine concentration in the grain. Although plant breeding is inherently a long-term process, more than two decades have now passed since the discovery of acrylamide in food, and progress towards low asparagine varieties remains slow. The metabolic interdependence of free asparagine with primary metabolism, nutrient balance and stress response presents a biological challenge, but also an opportunity for innovation at the interface of breeding, agronomy, and biotechnology:

i) the use of an advanced multi-disciplinary approach combining genetics, field phenotyping, metabolomics, and biotechnology could help to unravel the complex regulatory networks underlying free asparagine accumulation and identify novel candidate genes useful for advanced breeding programs;

ii) with the support of regulations governing the use of the technology, GM and GE could substantially accelerate the development of crops with reduced acrylamide-forming potential .

In conclusion, the obtaining of high-performing, low free asparagine genotypes offers a promising path toward reducing acrylamide-forming potential in cereal products while maintaining agronomic performance and quality. This multidisciplinary strategy ensures compliance with food safety regulations, fosters industry innovation, and promotes good health by developing safer and healthier food products aligned with sustainability goals.

CRediT authorship contribution statement

Elena Baldoni: Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Marco Napoli:** Writing – original draft. **Anna Szafrańska:** Writing – original draft. **Slađana Žilić:** Writing – original draft. **Delfina Barabaschi:** Writing – original draft. **Sonja Ilin:** Writing – original draft. **Andrea Tafuri:** Writing – original draft. **Antonio Pescatore:** Writing – original draft. **Beka Nešković:** Writing – original draft. **Viktor Korzun:** Writing – review & editing. **Tanya Curtis:** Writing – review & editing. **Nigel G. Halford:** Writing – review & editing, Writing – original draft, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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