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Effects of Fungicide Treatment on Free Amino Acid Concentration and Acrylamide-Forming Potential in Wheat

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Supporting Information

ABSTRACT: Acrylamide forms from free asparagine and reducing sugars during frying, baking, roasting, or high-temperature processing, and cereal products are major contributors to dietary acrylamide intake. Free asparagine concentration is the determining factor for acrylamide-forming potential in cereals, and this study investigated the effect of fungicide application on free asparagine accumulation in wheat grain. Free amino acid concentrations were measured in flour from 47 varieties of wheat grown in a field trial in 2011−2012. The wheat had been supplied with nitrogen and sulfur and treated with growth regulators and fungicides. Acrylamide formation was measured after the flour had been heated at 180 °C for 20 min. Flour was also analyzed from 24 (of the 47) varieties grown in adjacent plots that were treated in identical fashion except that no fungicide was applied, resulting in visible infection by *Septoria tritici*, yellow rust, and brown rust. Free asparagine concentration in the fungicide-treated wheat ranged from 1.596 to 3.987 mmol kg^{-1}, with a significant ($p < 0.001$ to $p = 0.006$, F test) effect of variety for not only free asparagine but all of the free amino acids apart from cysteine and ornithine. There was also a significant ($p < 0.001$, F test) effect of variety on acrylamide formation, which ranged from 134 to 992 $\mu g\ kg^{-1}$. There was a significant ($p < 0.001$, F test) correlation between free asparagine concentration and acrylamide formation. Both free asparagine concentration and acrylamide formation increased in response to a lack of fungicide treatment, the increases in acrylamide ranging from 2.7 to 370%. Free aspartic acid concentration also increased, whereas free glutamic acid concentration increased in some varieties but decreased in others, and free proline concentration decreased. The study showed disease control by fungicide application to be an important crop management measure for mitigating the problem of acrylamide formation in wheat products.

KEYWORDS: *acrylamide, asparagine, food safety, free amino acids, fungicide, processing contaminants, wheat*

INTRODUCTION

Acrylamide is a processing contaminant that forms within the Maillard reaction during the frying, baking, roasting, or high-temperature processing of mainly plant-derived foods. Here, we define a processing contaminant as a substance that is produced in a food when it is cooked or processed, but which is not present, or is present at much lower concentrations, in the raw, unprocessed, food and is undesirable either because it has an adverse effect on product quality or because it is potentially harmful.[1] Acrylamide is classed as a probable (Group 2a) human carcinogen by the International Agency for Research on Cancer,[2] on the basis of its action in rodents, and also has reproductive and neurotoxicological effects at high doses.[3]

The European Food Safety Authority (EFSA) Expert Panel on Contaminants in the Food Chain (CONTAM) stated in its 2015 report that the margin of exposure for acrylamide (defined as the ratio of the level at which a small but measurable effect is observed to the estimated exposure dose) indicates a concern for neoplastic effects based on the animal evidence.[4] As a result, the European Commission, which has already set "indicative" levels for the presence of acrylamide in food,[5] is reviewing its options for additional risk management measures. In the United States, the Food and Drug Administration (FDA) has so far stopped short of issuing advice or restrictions on levels of acrylamide in food, but it has issued an "action plan" with the goals of developing screening methods, identifying means to reduce exposure, assessing dietary exposure of American consumers, increasing understanding of acrylamide toxicology to enable quantitative risk assessment, and informing consumers.

Cereal products, including bread, crispbread, breakfast cereals, and biscuits are major contributors to dietary acrylamide intake, along with fried potato products and coffee.[6] It is extremely important, therefore, that the cereal supply chain addresses the acrylamide issue. The predominant route for acrylamide formation is via a Strecker-type degradation of free (i.e., soluble, nonprotein) asparagine by highly reactive carbonyl compounds produced within the Maillard reaction,[7−9] although other routes for its formation have been proposed, for example, with 3-aminopropionamide as a possible transient intermediate[10] or through pyrolysis of gluten.[11] The production of carbonyl compound intermediates within the Maillard reaction involves reducing sugars and other free amino acids, which means that the concentrations of these metabolites as well as free asparagine may affect acrylamide formation. However, in wheat (*Triticum aestivum*) and rye (*Secale cereale*), and probably other cereals, free asparagine concentration on its own closely correlates with acrylamide-forming potential.[12−16]

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The food industry responded rapidly to the discovery of acrylamide in its products, and the methods that have been devised to reduce acrylamide formation have been compiled in the Acrylamide Toolbox, published by Food Drink Europe,[17] a rare example of the food industry sharing knowledge to address a common problem, showing how seriously the industry takes the acrylamide issue. The challenge for the food industry is to reduce acrylamide levels while retaining the colors, flavors, and aromas that define products and brands and are demanded by consumers. This has been more successful for some food types than others, because many of the acrylamide mitigation tools show large variations in effectiveness across food categories. However, any method adopted by the food industry to reduce acrylamide formation in cereal products will be more effective from a low asparagine starting point, and it is important that wheat breeders and farmers respond to that if they are to retain market share.

Free asparagine concentration in many plant tissues responds to a range of environmental factors that are beyond the control of producers.[18,19] However, genetic factors also play a part, on their own and interacting with the environment (G × E),[12−16] whereas crop management, such as in the case of wheat ensuring that the crop is supplied with sufficient sulfur during cultivation, is absolutely critical.[12−14] Free asparagine will also accumulate in plants in response to pathogen infection,[18,20] and fungicide treatment in wheat has previously been shown to reduce free asparagine accumulation.[21] That study did not report acrylamide levels in heated flour and involved only three varieties, with an effect observed in 2006 apparently not evident in 2007. In this study, we investigated the effect of fungicide treatment as a potential crop management measure for controlling the acrylamide-forming potential of wheat using a much larger number of commercial varieties and measured effects not only on free asparagine but on other free amino acids as well.

MATERIALS AND METHODS

Chemicals. Ethanol (95% v/v, analytical grade) (Thermo Fisher Scientific UK Ltd., Loughborough, UK), HCl (Corning Life Science; supplied by Sigma-Adrich Co. Ltd., Poole, UK), and acrylamide-$^{13}C_3$ (Sigma-Adrich Co. Ltd.) were used. KOH for IC chromatography (Thermo Fisher Scientific UK Ltd.), amino acid standards (Phenomenex, Torrance, CA, USA), isotopically labeled amino acids (Cambridge Isotope Laboratories, Inc., Andover, MA, USA), and helium (high purity) (BOC Industrial Gases, Sheffield, UK) were also acquired.

Wheat Samples. Wheat grain samples for 47 varieties of winter wheat (*Triticum aestivum*) were provided by Saaten Union, Cowlinge, Suffolk, UK, from a field trial conducted in 2011−2012 at Ulceby, Lincolnshire, UK, postal code DN39 6DT, grid reference TA092142. The soil classification for this site is D-clay. The field trial comprised three replicate plots of each variety treated with fungicides and plant growth regulators from growth stages 30 to 65 (Table 1), and one plot of each variety treated with the same plant growth regulators but with no fungicides. Samples from only 24 of the untreated plots (varieties) were available. The grain samples were milled to fine, wholemeal flour for analysis.

Analysis of Free Amino Acid Concentrations. Flour (0.5 ± 0.005 g) was added to 10 mL of 0.01 N HCl and stirred for 15 min. The suspension was left to settle for 15 min at room temperature, and an aliquot (1.5 mL) was centrifuged at 7200*g* to produce a clear extract. Amino acids were derivatized using the EZ: Faast free amino acid kit (Phenomenex). Gas chromatography−mass spectrometry (GC-MS) analysis of the derivatized samples was carried out using an Agilent 6890 GC-5975-MS system (Agilent, Santa Clara, CA, USA) in electron impact mode, as described previously.[22] An aliquot of the derivatized amino acid solution (1 μL) was injected at 250 °C in split mode (20:1) onto a Zebron ZB-AAA capillary column (10 m × 0.25 mm; 0.25 μm film thickness). The oven temperature was held at 110 °C for 1 min and then increased at 30 °C min^{-1} to 310 °C. The transfer line and ion source were maintained at 320 and 230 °C, respectively; carrier gas flow rate was kept constant throughout the run at 1.1 mL min^{-1}. Amino acid standards were provided with the EZ: Faast kit and were >99% pure (Phenomenex). Separate calibration curves were calculated for each amino acid. The standards were also used before, during, and after the analysis of each batch of samples to check that the machine was running correctly. Analyses of the data (calibration curves and comparisons of standards over batches) were performed using the Agilent 5975 system data analysis software.

Table 1. Fungicides and Plant Growth Regulators Used in the Study[a]

date	growth stage	fungicide or plant growth regulator (PGR)	application rate (L ha^{-1})
March 25, 2012	30	Talius (fungicide) (DuPont (UK) Limited, Stevenage, UK)	0.15
		Ignite (fungicide) (BASF plc Crop Protection, Cheadle Hulme, UK)	0.75
		5C Cycocel (PGR) (BASF plc Crop Protection, Cheadle Hulme, UK)	1.25
April 26, 2012	32	Tracker (BASF plc Crop Protection, Cheadle Hulme, UK)	1.50
		Bravo 500 (Syngenta UK Limited, Fulbourn, Cambridgeshire, UK)	1.00
		Cyflamid (Certis UK, Great Abington, Cambridgeshire, UK)	0.25
May 8, 2012	37	Terpal (PGR) (BASF plc Crop Protection, Cheadle Hulme, UK)	1.00
May 20, 2012	45	Adexar (BASF plc Crop Protection, Cheadle Hulme, UK)	1.50
		Bravo 500 (Syngenta UK Limited, Fulbourn, Cambridgeshire, UK)	1.00
June 21, 2012	65	Proline 275 (Bayer CropScience, Cambridge, UK)	0.72
		Comet 200 (BASF plc Crop Protection, Cheadle Hulme, UK)	0.50

[a]The "treated" plots received all of the treatments shown, whereas the "untreated" plots received the plant regulators but not the fungicides.

Acrylamide Measurement. Flour samples (1.0 g) were heated for 20 min at 170 °C in unsealed glass vials (14 mL capacity) and analyzed by the analytical laboratory at PepsiCo Europe, Beaumont Park, UK. Acrylamide was extracted with 10 mL of water containing acrylamide-^{13}C as an internal standard. The solution was then purified by solid phase extraction with a proprietary sorbent phase followed by analysis by liquid chromatography−tandem mass spectrometry (LC-MS/MS). The method used was compatible with the Comité Européen de Normalization (European Committee for Standardisation) (CEN) standard method.

Statistical Analyses. There were three biological replicates (blocks in the field) for the fungicide-treated part of the trial and one biological replicate (block) for the untreated part. In each case there were two technical replicate samples of flour from each plot in each block and, for acrylamide only, two further technical replicates of each of these were taken. Analysis of variance (ANOVA) was applied to the amino acid and acrylamide data from the treated part of the trial, taking into account the design structure of plots within blocks and subsamples (technical replicates) from plots. The overall difference between varieties was therefore tested using the *F* test on the correct residual degrees of freedom (91) at the plot-to-plot level, and particular means (varieties) were compared using the standard error of the difference (SED) between means by way of the least significant

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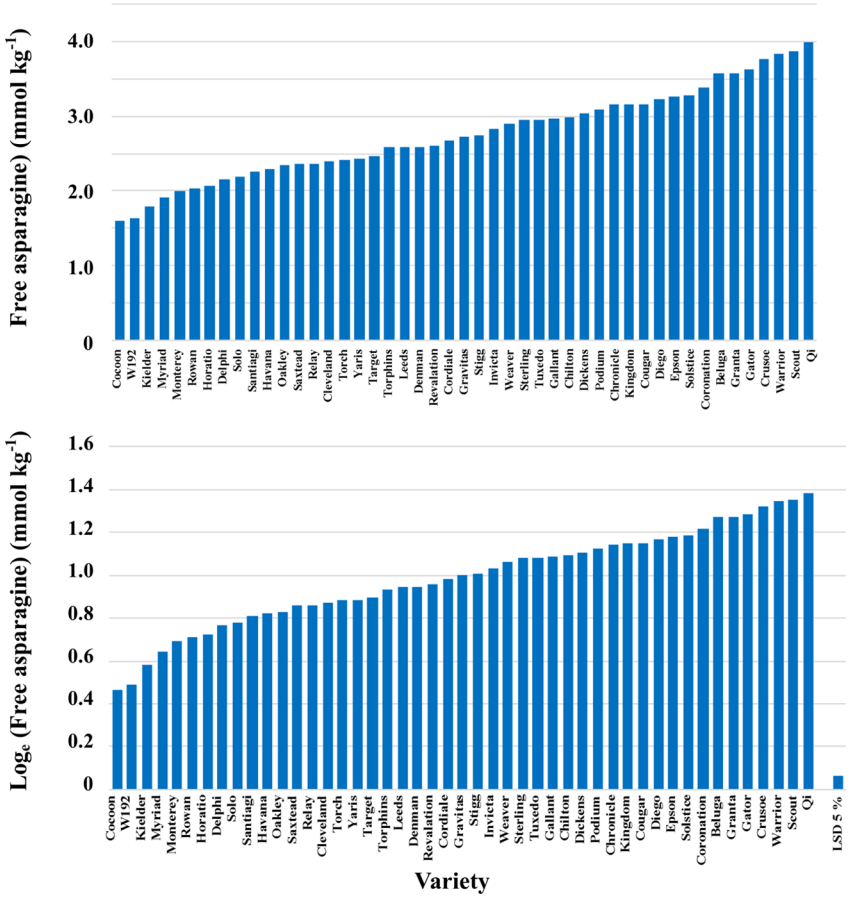

Figure 1. Graphs showing the mean (n = 3) free asparagine concentration on the raw scale (top) and $\log_e$ scale (bottom) in 47 varieties of wheat grown in a field trial in 2011−2012. The least significant difference (LSD) at 5% (on 91 degrees of freedom) is shown in the lower panel. The plants were treated with fungicides and growth regulators during cultivation (Table 1) and supplied with nitrogen (200 kg ha^{-1}) and sulfur (30 kg ha^{-1}). There was a significant (p < 0.001, F test) effect of variety on free asparagine concentration.

difference (LSD) at the 5% level of significance. A natural logarithmic transformation (to base e) was used to account for some heterogeneity of variance in the data over the varieties. Pearson correlation coefficient (r) was calculated between acrylamide and its main precursor, asparagine, and tested using the F test. As the untreated part of the trial was not replicated, the means of technical replicates per plot were calculated to allow comparison between varieties in this case, and thence difference in means was used to assess the effect of the treatment. Also, using the varietal means, the correlation for acrylamide and asparagine in treated versus untreated conditions was considered.

RESULTS AND DISCUSSION

Free Asparagine Levels in 47 Wheat Varieties and Acrylamide Formation in Heated Flour. Grain samples from 47 varieties of wheat were supplied by Saaten Union from a field trial at Ulceby, Lincolnshire, UK, in 2011−2012. The varieties were all on the UK's Agriculture and Horticulture Development Board (AHDB) Recommended List for commercial cultivation in that season. The field trial comprised four plots of each variety, three of which were treated with fungicides (Table 1) and the other one not. Otherwise, the plots were treated the same, receiving plant growth regulator treatment as well as fertilizer at a rate of 200 kg of nitrogen and 30 kg of sulfur per hectare.

The fungicide treatment program applied to the "treated" plots gave good disease control, and no disease was recorded above a 5% threshold, whereas the main disease in the "untreated" plot was *Septoria tritici* (*Mycosphaerella graminicola*) with some yellow rust (*Puccinia striiformis*) and brown rust (*Puccinia triticina*). The severity of the disease meant that grain could be harvested from only 24 of the 47 varieties.

The grain was milled to wholemeal flour and the flour analyzed for free amino acid content. The full data set is given in the Supporting Information, and the free asparagine levels for the 47 varieties growing in the "treated" plots are shown graphically in Figure 1. Free asparagine concentration ranged from 1.596 mmol kg^{-1} in variety Cocoon to 3.987 mmol kg^{-1} in Qi, an approximately 2.5-fold difference (Figure 1).

For statistical analysis, a natural log (to base e) transformation was used to account for some heterogeneity of variance. The full data set for all the free amino acids, with p values, standard error of the difference (SED), and LSD at the 5% level of significance are given in the Supporting Information, and the means for free asparagine are shown graphically with the LSD at 5% in Figure 1. The analysis showed there to be a significant effect of variety for almost all of the free amino acids, including free asparagine (p = 0.006 for isoleucine, otherwise p < 0.001, F test), the only exceptions being cysteine and the nonprotein amino acid, ornithine. The most abundant free amino acids were aspartic acid, asparagine, glutamic acid, and proline.

Acrylamide was measured after heating flour samples for 20 min at 180 °C and ranged from 134 μg kg^{-1} for Cocoon to 992 μg kg^{-1} for Qi, an approximately 7.4-fold difference (provided

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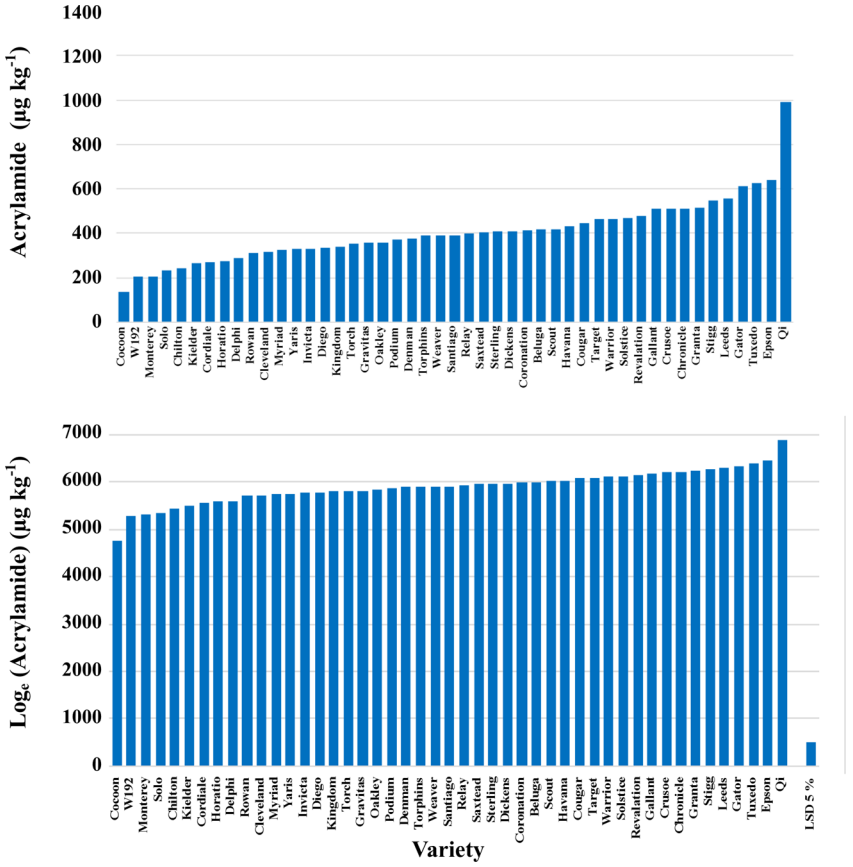

Figure 2. Graphs showing the mean (n = 3) level of acrylamide formation on the raw scale (top) and $\log_e$ scale (bottom) in heated flour (180 °C for 20 min) from 47 varieties of wheat grown in a field trial in 2011−2012. The plants were treated with fungicides and growth regulators during cultivation (Table 1) and supplied with nitrogen (200 kg ha^{-1}) and sulfur (30 kg ha^{-1}). The least significant difference (LSD) at 5% (on 91 degrees of freedom) is shown in the lower panel. There was a significant (p < 0.001, F test) effect of variety on acrylamide formation.

in full in the Supporting Information and shown graphically in Figure 2). The amount of acrylamide formed in flour from Qi was considerably greater than that for the next highest variety, Epson, the figure for which was 640 μg kg^{-1}, more than a third lower than Qi but still almost 4.8 times that of Cocoon. As with the free amino acid data, a $\log_e$ transformation was applied to enable valid statistical analysis. The $\log_e$-transformed figures are given in the Supporting Information and shown graphically in Figure 2, along with the LSD for 5% significance. There was a significant (p < 0.001, F test) correlation between free asparagine concentration and acrylamide formation (Figure 3), although the correlation coefficient of 0.557 was lower than in other data sets for wheat or rye.[12−16] One reason for this extra observed variation about the known relationship could be adverse climatic conditions in 2012, which was a particularly wet year in the United Kingdom.

It should be noted that these data come from one location and one harvest year, but they show clearly that commercial wheat varieties differ widely in their acrylamide-forming potential, consistent with the advice that variety selection could be a key element of acrylamide mitigation in wheat products.[23]

Effect of Fungicide Treatment. Free amino acid concentrations and acrylamide formation in heated flour in the "untreated" samples are given on the raw and $\log_e$ scales in the Supporting Information. The raw means for free asparagine concentration and acrylamide formation in treated versus untreated samples of the 24 varieties are shown graphically in

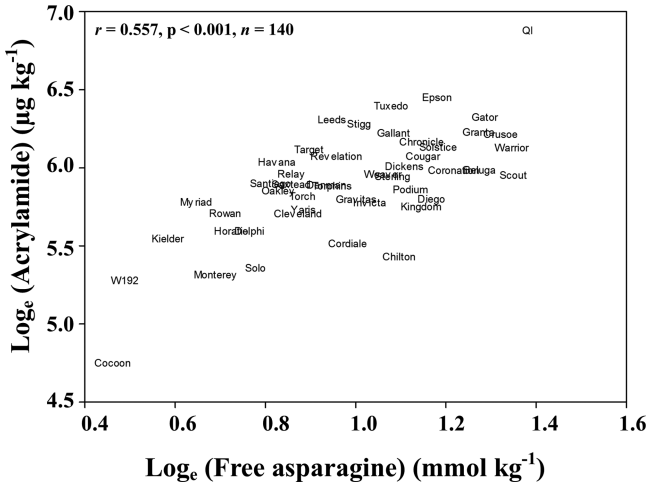

Figure 3. Plot showing the relationship on the $\log_e$ scale between free asparagine concentration and acrylamide formation in flour prepared from 47 varieties of wheat grown in a field trial in 2011−2012. Acrylamide was measured after the flour had been heated at 180 °C for 20 min. The Pearson correlation coefficient (r), p value (F test), and number of pairs of observations (n) are given on the graph. Plotted points are means (n = 3) for the varieties.

Figure 4, whereas the difference between the treated and untreated samples is shown in Figure 5. All of these varieties showed an increase in free asparagine concentration in response to a lack of fungicide treatment, with a concomitant effect on

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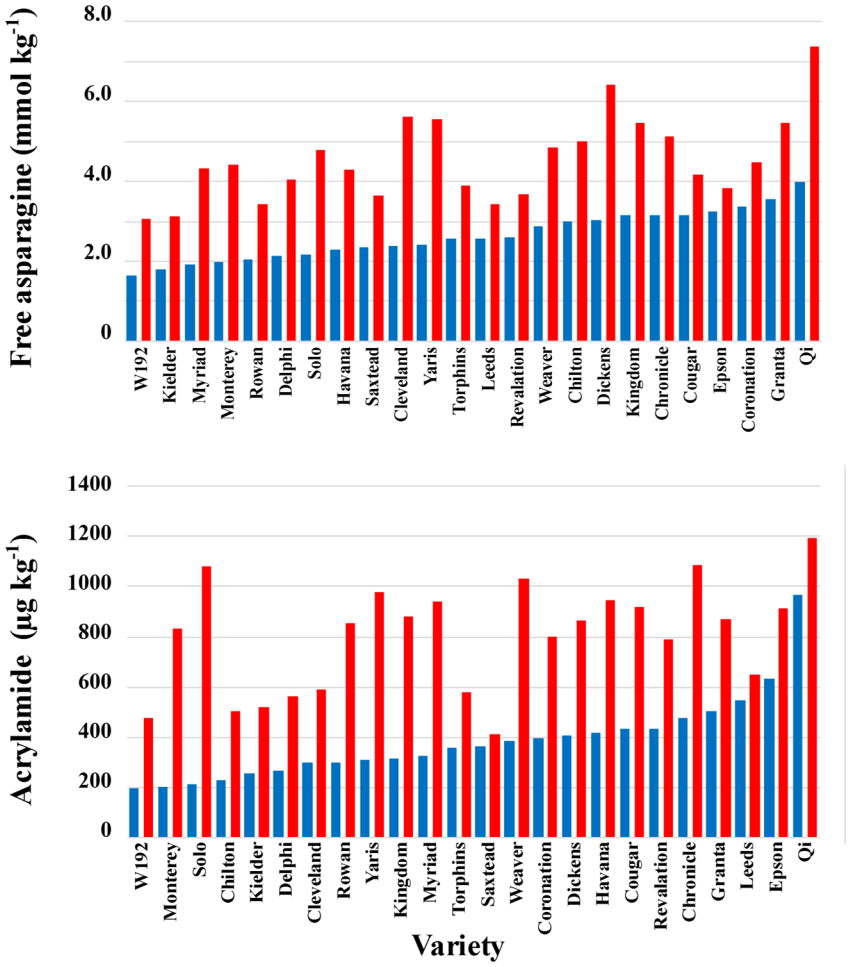

Figure 4. Graphs showing the mean free asparagine concentration (top) and acrylamide formation in heated flour (180 °C for 20 min) (bottom) on the raw scale in 24 varieties of wheat grown in a field trial in 2011−2012. The plants were all treated with growth regulators during cultivation (Table 1) and supplied with nitrogen (200 kg ha^{-1}) and sulfur (30 kg ha^{-1}), but plots were either treated with fungicides (Table 1) (blue, n = 3) or left untreated (red, n = 1).

acrylamide formation in heated flour. This resulted in all of the varieties being on the untreated side of the 1:1 relationship line when the treated and untreated means for free asparagine concentration and acrylamide formation were plotted against each other (Figure 6). However, there were big differences in the degree of change, with acrylamide formation increasing by 854 μg kg^{-1} (370%) in Solo but by only 11 μg kg^{-1} (2.7%) in Saxtead. This meant that the ranking of the varieties differed between the treated and untreated plots, illustrating the combined effects of genotype and crop management being strong, although it is noted that this interaction could not be assessed statistically as the untreated plots were not replicated.

There were also interesting effects for free aspartic acid, glutamic acid, and proline. As seen for free asparagine, aspartic acid increased in all of the varieties in response to a lack of fungicide treatment. Indeed, in general, the concentration of free aspartic acid increased by more than free asparagine, resulting in aspartic acid being the most abundant free amino acid in the untreated as well as treated condition. This contrasts with the effect of sulfur deficiency, which also causes increases in both of these free amino acids but with asparagine increasing more to become the most abundant free amino acid under sulfur deficiency.[12−14] Sulfur deficiency also causes an increase in free glutamine,[12−14] but little change was observed in the free glutamine concentration in response to a lack of fungicide treatment for the 24 varieties (greatest increase was 0.189 mmol kg^{-1} for Yaris).

The concentration of free glutamic acid showed a differential response, increasing in some varieties but declining in others in response to lack of fungicide treatment (Figure 5). Again, this contrasts with the effects of sulfur deficiency, which is associated with a rise in glutamic acid concentration.[12−14] Proline, on the other hand, decreased in all of the varieties in response to a lack of fungicide treatment (Figure 5), providing the starkest contrast with the effect of sulfur deficiency, which causes proline to increase.[12−14]

The increase in free asparagine concentration in response to a lack of fungicide treatment is consistent with results of a previous study showing asparagine synthetase activity to increase in wheat leaves in response to yellow rust infection:[24] Yellow rust was one of the diseases identified in the untreated plot in the present study, along with *Septoria tritici* and brown rust. Furthermore, treatment of wheat with a mycotoxin, deoxynivalenol (DON), produced by another pathogenic fungus, *Fusarium graminearum*, which causes Fusarium head blight, has also been shown to increase the levels of free asparagine and aspartic acid in the grain,[20] although in that case free glutamine increased as well. Wheat has four classes of asparagine synthetase genes, *TaASN1*, *TaASN2*, *TaASN3*, and *TaASN4*,[25] but it is not known which of them was responsible

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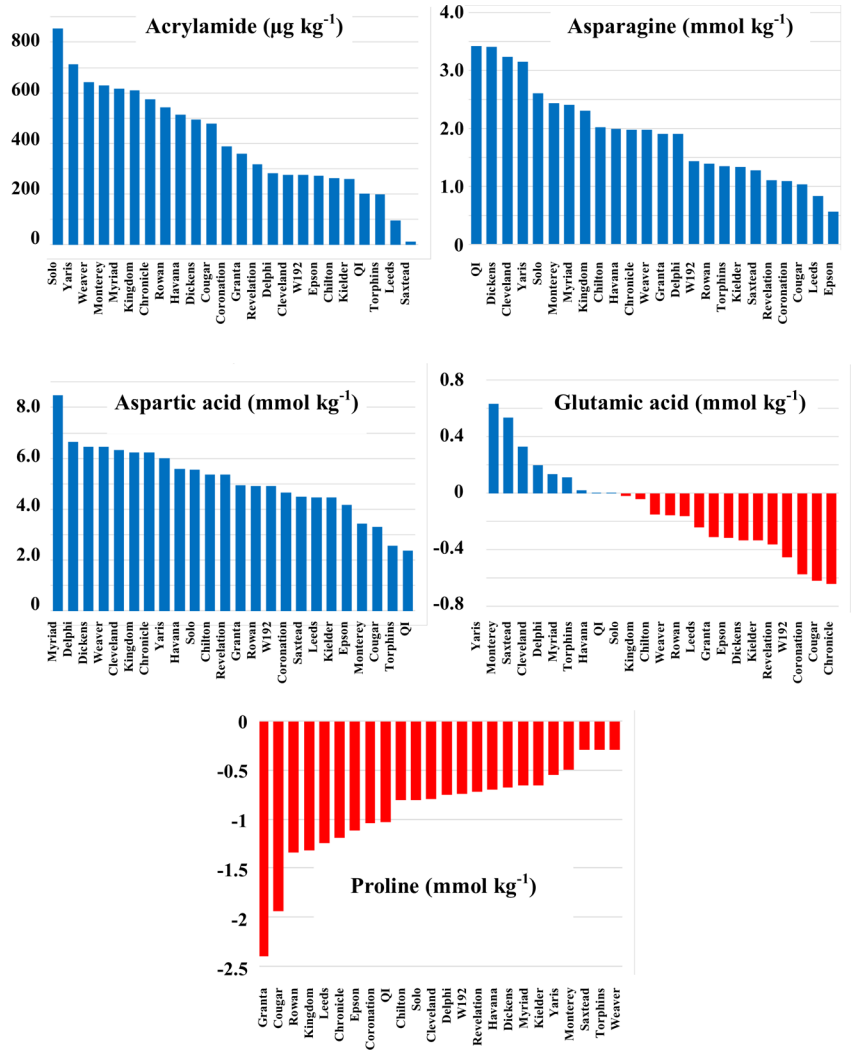

Figure 5. Graphs showing the difference in means between flour from fungicide-untreated (n = 1) and -treated (n = 3) plots of wheat grown in a field trial in 2011−2012. The plants were all treated with growth regulators during cultivation (Table 1) and supplied with nitrogen (200 kg ha^{-1}) and sulfur (30 kg ha^{-1}), but plots were either treated with fungicides (Table 1) or left untreated. A positive value (shown in blue) indicates that the concentration in the flour from the untreated plot was higher than that in the treated plot, whereas a negative value (red) indicates the opposite. Data are shown for acrylamide formed in heated flour (180 °C for 20 min), free asparagine, free aspartic acid, free glutamic acid, and free proline, as indicated.

for the increase in asparagine synthetase activity in response to yellow rust.[24] An asparagine synthetase (CaAS1) has also been shown to be essential for pathogen defense in pepper (*Capsicum annuum*).[26]

Relatively little is known about the signaling mechanisms involved in the response of free asparagine concentration to pathogen infection, but clues are starting to emerge. One candidate signaling factor is the general control non-derepressible-2 (GCN2)-type protein kinase,[27] which has been shown to affect asparagine synthetase gene expression in wheat seedlings.[28] Yeast GCN2 has 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.[29]

A second candidate is another protein kinase, sucrose nonfermenting-1-related protein kinase-1 (SnRK1). SnRK1 coordinates nutrient availability and stress and hormonal signaling, maintains cellular energy balance,[27,30] and is directly implicated in the control of asparagine synthetase gene expression in *Arabidopsis thaliana*.[30] Recently, it has been shown to interact with the protein encoded by *TaFROG*, a gene induced in wheat by both *Fusarium* infection and DON treatment,[31] and to became activated in wheat in response to *Septoria tritici* infection.[32]

Implications for Commercial Wheat Production. The study showed fungicide application and, by inference, crop disease to have a profound effect on the free asparagine concentration and acrylamide-forming potential of grain from a range of commercial wheat varieties. We propose that effective disease control through fungicide application be adopted as a second crop management measure, alongside ensuring sulfur sufficiency, to mitigate the problem of acrylamide formation in wheat products. We also recommend that regulatory authorities take the consequences for acrylamide-forming potential into account when considering the risks and benefits of fungicide usage.

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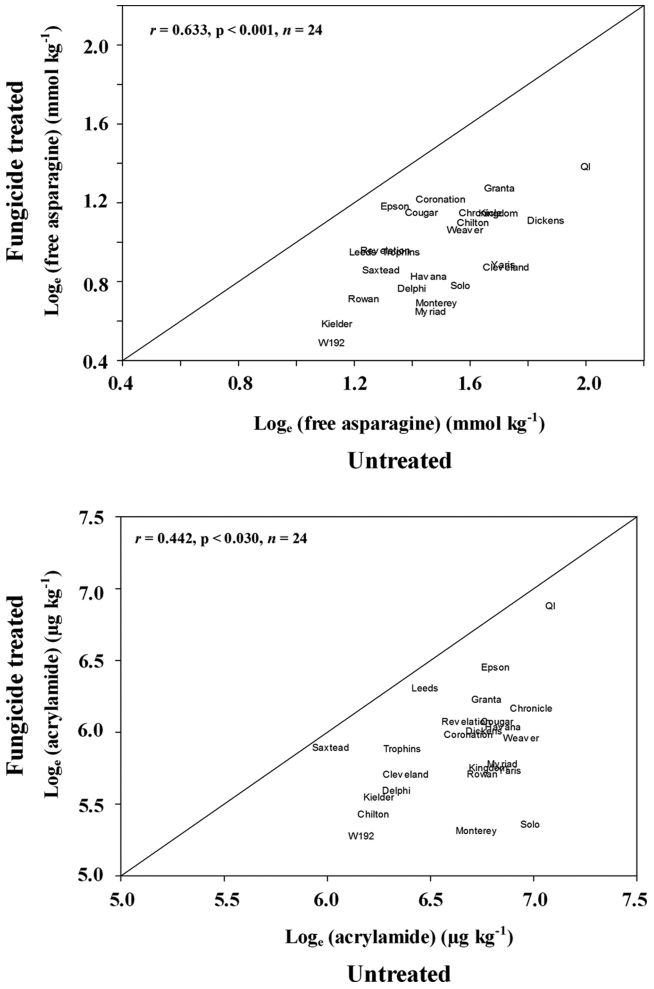

Figure 6. Plots showing concentrations of free asparagine (top) and acrylamide formed in heated flour (180 °C for 20 min) (bottom) in flour from fungicide-untreated and -treated plots of wheat grown in a field trial in 2011−2012. The plants were all treated with growth regulators during cultivation (Table 1) and supplied with nitrogen (200 kg ha^{-1}) and sulfur (30 kg ha^{-1}), but plots were either treated with fungicides (Table 1) or left untreated. The 1:1 line is shown, and Pearson correlation coefficient (r), p value (F test), and number of pairs of observations (n) are given on the graphs. The plotted points are means for the varieties in the fungicide-treated (n = 3) versus untreated (n = 1) condition.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b04520.

Data showing free amino acid concentrations and acrylamide formed in heated flour (XLSX)

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Notes

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