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# Glyphosate Applied Preharvest Induces Shikimic Acid Accumulation in Hard Red Spring Wheat (*Triticum aestivum*)

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Glyphosate is a nonselective herbicide used as a harvest aid in a variety of crops. Glyphosate is absorbed into the foliage and translocated to metabolically active regions in the plant where it interferes with the shikimic acid pathway. Experiments were conducted to determine the accumulation and distribution of shikimic acid in wheat treated with glyphosate at soft and hard dough stages of kernel development and to determine the fate of shikimic acid during milling and bread making. Elevated levels of shikimic acid were detected throughout the wheat plant. Shikimic acid concentrations peaked 3−7 days after treatment and then declined until harvest. Shikimic acid content was 3-fold greater in flour and 2-fold greater in the bread derived from treated wheat than nontreated wheat. Similarly, elevated levels of shikimic acid were found in the crumbs and crust of bread made with flour from glyphosate treated wheat. Glyphosate applied preharvest resulted in shikimic acid accumulation in hard red spring wheat and subsequent end-use products.

**KEYWORDS: Shikimic acid; preharvest glyphosate; wheat**

## INTRODUCTION

Herbicides can be applied prior to harvest as a method to control weeds and accelerate crop dry-down, which promotes timely and efficient harvest. Several herbicides are registered in wheat as harvest aids, including glyphosate, *N*-(phosphonomethyl)glycine, 2,4-D, (2,4-dichlorophenoxy)acetic acid, and metsulfuron, methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]benzoate.

Glyphosate is a nonselective herbicide that is commonly used as a harvest aid in a variety of crops. Glyphosate can be applied to wheat when the kernel is at the hard dough (HD) stage but can be applied at least 7 days prior to harvest. Glyphosate is absorbed into the foliage and translocated to the active growing regions of the plant where it interferes with the shikimic acid pathway by competitively inhibiting 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase (*1*). The shikimic acid pathway is used by plants, fungi, and microorganisms for synthesis of essential aromatics, including L-α-amino acids (Phe, Tyr, and Trp). These amino acids are important in protein synthesis and as precursors to the folate coenzymes and several isoprenoid quinones (*2*).

Approximately 20% of the carbon fixed by plants is normally channeled through the shikimic acid pathway (*3*). Inhibition of EPSP synthase deregulates the pathway, which results in uncontrolled flow of carbon and the subsequent massive accumulation of shikimate or shikimate-3-phosphate in affected plant tissue (*4*). Accumulation of shikimate results in increased production of derived shikimate benzoic acids, such as protocatechuate and gallate acids in plants (*3*, *4*). Concentrations of vanillic and syringic acids, which are methylated forms of protocatechuic and gallic acids, respectively, were not affected by glyphosate treatment (*5*).

Elevated concentrations of phenolic acids are of interest because of their potential oxidation properties. Protocatechuic, *p*-hydroxybenzoic, gentisic, caffeic, chlorogenic, syringic, *p*-coumaric, and ferulic acids are phenolic compounds in wheat that have antioxidant activity (*6*). Sawa et al. (*7*) reported that vanillic, gallic, and shikimic acids exhibited remarkably high free radical scavenging activity. Total phenolic acid content in wheat flour ranges from 71 to 180 ppm, with *trans*-ferulic, syringic, and vanillic acids being the principle phenolic acids (*6*, *8*). Naturally occurring antioxidants may act to terminate free radical chain reactions in biological systems and provide health benefits to the consumer, while prooxidants may not provide such health benefits and be detrimental to human health (*9*, *10*).

Phenolic acids could be important in determining the end-use quality of wheat. Phenolic acids typically found in wheat have been associated with dough weakening (*11*). Ferulic acid is thought to weaken dough by reducing disulfide bonds, thus disrupting interchain disulfide bonds during dough development (*12*). Preliminary research on hard red spring wheat indicates that glyphosate applied preharvest (≥30% grain moisture) can affect gluten and dough properties (*13*). Flour from wheat treated

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with glyphosate had greater gluten index values, longer dough development time, and greater dough stability than flour from untreated wheat. Darwent et al. (*14*) reported that more energy was required to develop dough made with flour from wheat treated with preharvest glyphosate as compared to nontreated wheat. The objectives of this research were to determine the concentration and location of shikimic acid accumulation within treated wheat plant and to determine the fate of shikimic acid during milling and bread making.

## MATERIALS AND METHODS

**Wheat Samples.** Wheat samples used to determine the accumulation and distribution of shikimic acid in wheat treated with glyphosate were obtained from a field research project conducted near Fargo, ND, in 2002. Treatments included an untreated control and glyphosate at 0.84 kg acid equivalent (ae)/ha as the isopropylamine salt applied with ammonium sulfate at 0.70 kg/ha in 80 L/ha spray volume. Treatments were applied to Cv. Alsen hard red spring wheat at the soft dough stage (SD) and HD stage. Glyphosate is registered for application to HD stage wheat or at least 7 days prior to harvest. The HD stage was used as the commercial standard. The SD stage was included as an extreme end point for the desired effect of increasing shikimic acid content. Kernel moisture content averaged 45% at SD and 33% at HD. Kernel moisture was determined by randomly collecting 10 spikes per replication. Spikes were weighed and placed in a forced air oven at 135 °C for 2 h. Spikes were allowed to cool to room temperature and reweighed. Preliminary experiments indicated no change in weight after 2 h of drying. Spike moisture content and kernel moisture content were considered equivalent. Thomas et al. (*15*) determined that during late grain filling and early ripening, the moisture contents of the entire spike and kernel were similar.

Ten plants were sampled from each plot prior to treatment and 3, 7, and 21 days after treatment for plots treated at the SD stage and 3, 7, and 10 days after treatment for plots treated at the HD stage. Plants were divided into roots, shoots, leaves, flag-leaf, peduncle, rachis, and kernels and stored at −4 °C until time of shikimic acid analysis.

A second experiment was conducted to determine the shikimic acid content of flour and bread made from wheat treated with herbicides applied preharvest. Grain samples were obtained from a field experiment conducted near Langdon and Prosper, ND, in 1999 and 2000. Herbicide treatments included two formulations of glyphosate at 0.84 kg ae/ha, metsulfuron at 0.08 kg ai/ha, plus 2,4-D at 1.26 kg ae/ha and an untreated control applied to hard red spring wheat Cv. Parshall at SD and HD stages. The two glyphosate formulations were the isopropylamine and trimethyl-sulfonium salts. The grain was harvested at maturity with a small plot combine and dried to ∼12% moisture in forced-air dryers at 40 °C.

The grain was cleaned, tempered to 15.5% moisture for 24 h, and milled into straight-grade flour on a Buhler laboratory mill. A sample of flour from each treatment was stored at 4 °C until the time of shikimic acid extraction and analysis. The flour was made into bread using a straight dough procedure based on approved method 10-09 (AACC 2000). All samples were mixed to optimum development until a thin membrane was formed and was translucent when stretched. Optimum dough was calculated as the water absorption of flour obtained on the farinograph minus 1.5%, which resulted in better machinability and reduced stickiness for molding and sheeting.

The baking formula (flour basis) was 100.0 g of flour (14% mb), 5.0 g of sugar, 2.0 g of salt, 3.0 g of shortening, 1.0 g of vacuum-packed yeast, 0.1 g of ammonium phosphate monobasic, 0.1 g of fungal α-amylase (17 SKB units, American Ingredients, Co., Kansas City, MO), and 20 ppm of ascorbic acid. A two step punching procedure was adopted using 180 min for fermentation. All doughs were proofed at 30 °C for 55 min at 85% relative humidity before baking. Dough pieces were baked at 220 °C for 25 min. Bread loaves were allowed to cool and then frozen until time of shikimic acid extraction.

**Shikimic Acid Extraction.** Plant material, flour, crust, and crumbs of bread were macerated with a mortar and pestle in 0.25 HCl (1:3 weight/volume) (*16*). The liquid and ground materials were transferred to centrifuge tubes and were centrifuged at 1200*g* for 10 min. The supernatant was removed for analysis by high-performance liquid chromatography (HPLC).

**Table 1.** Shikimic Acid Content (ppm) in Root, Stem, Leaf, Kernel, and Rachis of Hard Red Spring Wheat Following Treatment with Glyphosate at SD and HD Stage

| dough stage | *d* [a] | root | stem | leaf | kernel | rachis |
|---|---|---|---|---|---|---|
| soft | 0 | 0 | 0 | 0 | 32 | 3 |
| | 3 | 89 | 30 | 67 | 105 | 173 |
| | 7 | 368 | 267 | 79 | 122 | 246 |
| harvest | 21 | 8 | 22 | 13 | 28 | 12 |
| LSD (0.05) | | 215 | 278 | 34 | 77 | 46 |
| hard | 0 | 5 | 2 | 0 | 6 | 0 |
| | 3 | 312 | 180 | 125 | 58 | 232 |
| | 7 | 313 | 256 | 38 | 10 | 42 |
| harvest | 10 | 81 | 120 | 21 | 3 | 8 |
| LSD (0.05) | | 54 | 64 | 105 | 55 | 199 |

[a] *d*, days after treatment.

**HPLC Analysis.** A 250 $\mu$L aliquot of the supernatant of the crude extract described above was combined with the HPLC mobile phase (1:3 dilution). The shikimic acid concentration was determined using a Hewlett-Packard model 1050 HPLC (Hewlett-Packard, Inc., Palo Alto, CA) equipped with a UV detector at a wavelength of 213 nm. Liquid chromatography separation was performed on a Phenomenex Lichrosorb 5 $NH_2$ column (240 mm × 4 mm) with a mobile phase of 95% acetonitrile, 1% phosphoric acid, and 4% water at a flow rate of 1 mL/min. The sample injection volume was 20 $\mu$L. The retention time of the shikimic acid was approximately 8 min.

A linear calibration curve based on peak area was generated using known shikimic acid concentrations ranging from 0.01 to 5 $\mu$g/mL. The stock solution of shikimic acid ([−]3α,4α,5β-trihydroxy-1-cyclohexene-1-carboxylic acid) consisted of analytical grade herbicide (Sigma Chemical Co., St. Louis, MO, chemical purity >99%) dissolved in milli-Q water. The concentration of shikimic acid from wheat seed, ground wheat seed, crust, crumb, and whole bread samples was determined by entering peak area values into a regression equation describing the calibration curve. The minimum calibration concentration was 100 ppb. The concentration of shikimic acid was expressed as ppm on a dry weight basis of ground material.

**Statistical Analysis.** The experiment was in a randomized complete block design with four replicates. Data were analyzed using the Statistical Analysis System (SAS Institute, Cary, NC). Data were subjected to an analysis of variance across environments, since the variances among environments were homogeneous. Fisher's Protected LSD separated means at the 5% level.

## RESULTS AND DISCUSSION

Prior to treatment with glyphosate, shikimic acid was detected only in trace amounts at the SD stage in the kernel and rachis and in the root, stem, and kernel of wheat at the HD stage (**Table 1**). Shikimic acid was found in all plant parts 3 days after treatment at both the SD or HD stages. Three days after treatment, all plant parts had 98−100% increased shikimic acid content, except the kernel that had an increase of 70% at the SD stage and 90% at the HD stage.

Shikimic acid content 7 days after treatment tended to be greater in the root, stem, and leaves at the SD stage than at the HD stage. Concentration of shikimic acid continued to increase in plant roots and stems, while shikimic acid in the leaves, kernel, and rachis decreased from 3 to 7 days after treatment. Shikimic acid concentrations at harvest in the various plant parts decreased as compared to 7 days after treatment with the highest concentration occurring in the stem and kernel when treated at the SD stage and in the stem when treated at the HD stage.

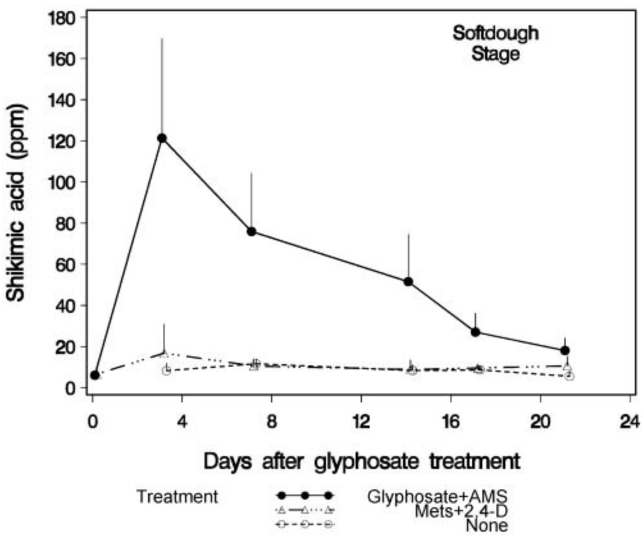


**Figure 1.** Shikimic acid content (ppm) in hard red spring wheat after preharvest glyphosate treatment.

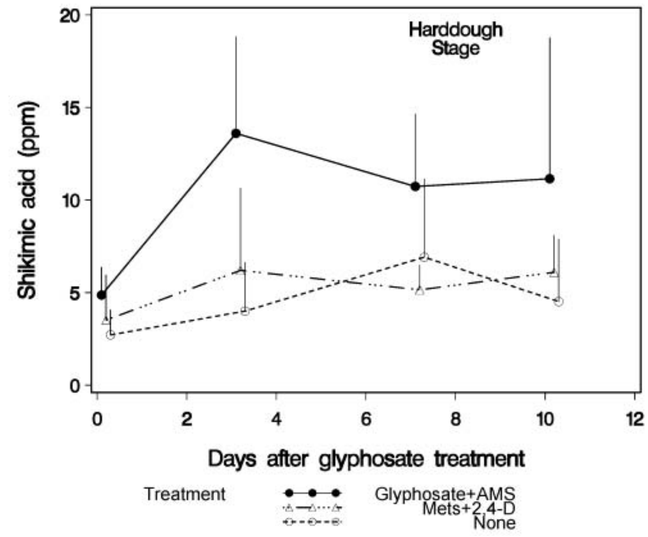


**Figure 2.** Shikimic acid content (ppm) in hard red spring wheat after preharvest glyphosate treatment.

Wheat treated with glyphosate at the SD stage is less physiologically mature than wheat treated at the HD stage. Physical maturity might affect translocation of glyphosate throughout the plant and/or affect the rate of accumulation of shikimic acid in plant tissues. Before treatment, shikimic acid content in the kernel was greater at the SD than at the HD stage (**Table 1**). Shikimic acid concentration 3 days after treatment at the SD stage were highest in the kernel and rachis, whereas wheat treated at the HD stage had high concentrations in all plant parts. Shikimic acid concentrations in the stem and root continued to increase up to 7 days after treatment at both the SD and the HD stages and then began to decline. Variability in shikimic acid accumulation could be due to relative ability of tissue to metabolize and/or translocate shikimic acid.

Neither metsulfuron nor 2,4-D is known to affect the shikimic acid pathway (*15*, *16*). These herbicides were included as treated controls, because they are commonly used for weed control in wheat prior to harvest.

Glyphosate applied at the SD stage increased the shikimic acid content of wheat kernels 3 days after treatment from 5 to 120 ppm, a 24-fold increase (**Figure 1**). However, shikimic acid levels 21 days after treatment had decreased to ~15 ppm. Shikimic acid levels in wheat kernels treated with glyphosate at the HD stage 3 days after treatment had increased steadily from a low of 5 ppm to a high of 15 ppm (**Figure 2**). The shikimic acid concentration by day 12 remained level at ~12 ppm. The highest concentration at the SD stage was 8-fold greater than the highest concentration at the HD stage; however, both stages after treatment had similar shikimic acid concentrations at harvest of 12−15 ppm. These results are similar to those found in the previous experiment (**Table 1**). Shikimic acid content in wheat kernels did not change when kernels were treated with metsulfuron plus 2,4-D at either the HD or the SD stage (**Figures 1** and **2**).

Elevated levels of shikimic acid were found in flour and bread made from wheat treated with glyphosate (**Table 2**). Flour contained ~57% less shikimic acid than did the kernel, regardless of treatment. Still, the amount of shikimic acid in flour from wheat treated with glyphosate-ipa and glyphosate-tms was 3 and 2.5 times greater when compared with flour from untreated wheat and from wheat treated with metsulfuron+2,4-D, respectively. Both the wheat kernel and the flour had 3-fold more shikimic acid from glyphosate treated plants than untreated plants.

**Table 2.** Shikimic Acid Content (ppm) in Kernel, Flour, Crumb, Crust, and Bread from Hard Red Spring Wheat Following Preharvest Treatment with Glyphosate

| treatment[a] | kernel | flour | crumb | crust | bread |
|---|---|---|---|---|---|
| glyphosate-ipa | 99 | 41 | 20 | 30 | 24 |
| glyphosate-tms | 94 | 40 | 16 | 24 | 19 |
| metsulfuron + 2,4D | 40 | 17 | 9 | 12 | 10 |
| control | 32 | 14 | 10 | 12 | 11 |
| LSD (0.05) | 29 | 8 | 4 | 4 | 4 |

[a] Glyphosate-ipa is the isopropylamine salt and glyphosate-tms is the trimethylsulfonium salt.

Bread made from wheat treated with glyphosate-ipa and glyphosate-tms resulted in more shikimic acid in both the crumb and the crust when compared to the untreated wheat crumb and crust. Crumbs from glyphosate-ipa treated wheat kernels had shikimic acid levels 50% higher than untreated wheat crumbs. The crust from glyphosate-ipa treated wheat kernels contained 60% more shikimic acid as compared to the untreated crust samples. Crust and crumbs from glyphosate-tms treated wheat also exhibited similar increases, but no increases were seen in the crust and crumbs from wheat treated with metsulfuron plus 2,4-D. The final consumable bread product processed from treated glyphosate wheat flour showed that shikimic acid content increased on average 49% when compared to the bread produced from untreated wheat. Shikimic acid content of bread made from wheat treated with metsulfuron plus 2,4-D was similar to the untreated bread product.

Mature kernels of wheat treated with preharvest glyphosate at both physiological stages had greater shikimic acid concentrations than from untreated wheat. This pattern continued to the flour and finally to the end-use bread product. Wheat treated with metsulfuron plus 2,4-D at both physiological stages showed no increase in shikimic acid concentration in the kernel and final consumable bread product when compared to the untreated kernel.

Phenolic acids such as shikimic acid have antioxidant, possibly prooxidant, and free radical scavenging activities, which may be important in human nutrition. More research is necessary to determine kernel composition changes and changes in end-use products when glyphosate is applied preharvest in maturing wheat.

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