### Title

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# Metabolomics Response of Wheat (*Triticum aestivum*) to "Green" and Conventional Nonionic Surfactants at Different Application Stages

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**ABSTRACT:** Physiological, nutritional, and metabolomic responses of wheat (*Triticum aestivum*) plants to two surfactants (SAs) (nonylphenoxy polyethoxy ethanol at 1 g/L—"Nonionic SA" versus a combination of 0.5 g/L xanthan gum and 5 g/L triethyl citrate—"Green SA") were investigated at two application stages and three plant response times (e.g., day 2, day 4, and day 8). The concentration was based on the manufacturer's recommendation. Although dry biomass and mineral nutrients remained unchanged for most experimental conditions, metabolomics revealed changes in plant internal status. When the Green SA was applied at the early tillering stage (ET, day 21), cysteine and methionine metabolism was consistently perturbed for all three plant response times. However, metabolite reprogramming faded rapidly by day 8, with only one significantly altered amino acid (aspartic acid) detected. On the contrary, when SAs were applied at the flag leaf stage (FL, day 32), the maximum perturbation of metabolomic pathways (10 pathways perturbed for Green SA and 8 for Nonionic SA) occurred on day 8 with a significant perturbation of the tricarboxylic acid cycle for both SAs. Furthermore, Green SA applied at FL disturbed more metabolomic pathways and almost two times more metabolites (19 vs 10) that were positively correlated to the plant response time than Nonionic SA. That indicated Green SA applied at FL resulted in a more profound impact on the plant defense system and nitrogen and carbon metabolism, mostly increasing the levels of perturbed metabolites by 1.1- to 2.0-fold changes. Determining the molecular response of plants after SA application can serve to better design targeted delivery of nutrients or active ingredients onto superhydrophobic leaf surfaces.

**KEYWORDS:** *metabolites, pathway analysis, agriculture, surfactants, plant response*

## INTRODUCTION

Due to the hydrophobic nature of plant leaf surfaces, surfactants (SAs) are often adopted to help deliver active ingredients of agrochemicals into plants. Depending on the structure of leaf epicuticular wax and trichome density, the water contact angle could be as high as 150 °C, causing sprayed droplets to easily run off or even be repelled.[1] Under such circumstances, SAs are used to assist spray application to deliver fertilizers,[2,3] pesticides,[4,5] and microbial inoculants.[6,7] SAs are classified as anionic, cationic, nonionic, and amphoteric based on the nature of hydrophilic group properties.[3] Many common SAs are derived from petrochemicals. Due to ecological, environmental, and toxicological concerns, there is a need for seeking and developing alternative SAs from renewable sources.[3,8] Many natural products have been considered for this purpose, such as vegetable oils,[9] lecithin,[10] sugars,[11] amino acids,[12] and chitosan.[13]

Past surfactant-related studies in agriculture area were mainly focused on the physical interaction between the surfactant and leaf surfaces,[14−17] assisting conventional/nanosized agrochemical applications,[2,6,18] potential toxicity,[3,19,20] environmental impacts,[3,21] and formulation of new alternative SAs.[3,22] For example, two commercial SAs (LI 700@ and Agral@), two pure SAs (Genapol@ X-080 and Triton 100-X), and one humectant (glycerol) were employed to assist phosphorous (P) translocation in wheat through foliar applications.[2] Glycerol resulted in less than 30% of P uptake by wheat, but the other SAs increased P uptake by more than 70%. In particular, Triton 100-X led to 82.4 and 83.5% increased P uptake when it was applied at the early tillering (ET) and flag leaf (FL) emergence period. In another short-term foliar exposure study, 0.05 weight (wt) % Tween20 was used to deliver zinc salts and Zn nanomaterials (NMs) into wheat leaves.[23] Applying zinc salts to wheat led to about six times greater Zn content in wheat leaves than those exposed to ZnO NMs, while no significant differences were found among ZnO NMs with different coatings. The authors proposed that ionic adsorption was likely the dominant pathway though the cuticular pathway. Most of the above-mentioned studies only considered the surfactant as a wetting agent, however, plant molecular response caused by the surfactant has seldomly been examined.[4] Furthermore, most studies examined plant response at one single time, instead of considering plant response at various times after the exposure assay.

Metabolites are the end products of plant regulatory processes at the molecular level. In addition to physiological

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phenotypic expression, metabolomics allows to distinguish and quantify molecular level changes within plants and the rhizosphere.[4,24−26] However, only a few studies have conducted a quantitative metabolite comparison (targeted metabolomics),[4,24,27−29] while other studies were either semiquantitative (untargeted)[30−32] or evaluated the total content of only certain metabolite groups (e.g., sugars, phenolics, nonenzymatic antioxidants, etc.).[33,34] A recent study employed the commercial surfactant TritonX-100 to improve the foliar application of $Cu(OH)_2$ NMs and $MoO_3$ NMs to early-stage wheat seedlings.[4] Six groups of metabolites were considered, for a total of 83 metabolites. Metabolomics revealed that surfactant application significantly promoted NM interactions with wheat leaves. However, even applying TritonX-100 alone (with no NMs) resulted in significant metabolite reprogramming, despite minor changes in physiological and nutrient parameters. Leucine, lysine, phenylalanine, proline, serine, tryptophan, linolenic acid, adenosine, guanosine, uridine, and trehalose had more than 5 times fold changes in wheat leaves and roots. Metabolomics further demonstrated the favoring of N metabolism over C metabolism, potential membrane lipid peroxidation, and perturbation of pyrimidine and purine metabolism to defend against stress.[4]

In the current work, one "Green" surfactant (Green SA) and one "Conventional" surfactant (Nonionic SA) were applied to wheat seedlings through foliar exposures at ET and FL stages. A mixture of xanthan gum and triethyl citrate was considered an alternative "green" surfactant agent. The polysaccharide xanthan gum can be generated via microbial fermentation, and has high biodegradability and compatibility with other chemical agents.[35,36] Triethyl citrate is an ester of naturally derived citric and has been investigated as an adjuvant in agriculture.[37] This Green SA has shown the potential to deliver active ingredients into plant surfaces by increasing wettability and moisture retention times.[36] A full life-cycle assessment would be needed to determine the environmental benefits of the Green SA over more conventional SAs, but that is beyond our current scope. Our objective here is to quantify plant response to SAs at a molecular level. Sample harvesting was conducted at various time intervals after the surfactant application, to monitor plant responses with time. Morphological, physiological, and biochemical data were collected, specifically, and metabolite content changes within wheat leaves were measured using liquid chromatography (LC)−mass spectrometry. To cover the majority of metabolites participating in the main metabolomic pathways, 91 targeted metabolites were considered. Findings from this study provide valuable insights into plant responses to SAs at the molecular level, which is of great importance given the wide application of SAs in agriculture. Identifying significantly altered metabolomics followed by integration of pathway analysis is helpful to illustrate the internal plant response mechanism. Following the metabolomics response at different time intervals provides an understanding of the persistence of metabolic reprogramming.

## MATERIALS AND METHODS

**Seed Germination and Plant Growth Conditions.** Wheat (*Triticum aestivum* "Red Fife"), a common crop with superhydrophobic leaf properties, was selected as a representative crop in the current study. First, wheat seeds were disinfected with 1% sodium hypochlorite solution (*Supelco*. Product No. XX06373-76) followed by 5−10 times rinsing with deionized water (DI). Then the wheat seeds were left soaking in a beaker with Nanopure water for 24 h. At the end of the immersion period, sets of four seeds were germinated in a premoisturized small pot with approximately 40 g of 50% vermiculite and 50% perlite. Seeds were germinated with spacing in between and at around 40 to 50 mm in depth. A 10% Hoagland water was used throughout the experiment, to provide sufficient macro- and micronutrients during early plant growth stages. Growth conditions were as follows: 16 h of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and 8 h under dark per day; temperature and relative humidity were controlled at around 22 °C and 60%, respectively. Water content in the pots was maintained between 70 and 90% during the entire experimental period. Five days after germination, similar-sized wheat seedlings were selected and transplanted into new pots, with two plants per pot. Foliar exposures were conducted once for a given treatment, but at two different plant growth stages: day 21 corresponding to the wheat ET stage and day 32 corresponding to the wheat FL collar visible stage.[2] Eight replicates were conducted across three treatments (Control, Green SA, and Nonionic SA), two applied stages (ET and FL), and three plant response times (2, 4, and 8 days). To account for all experimental conditions and testing needs, 145 pots were utilized with a total of 290 plants. Due to the superhydrophobic characteristics of the wheat leaf surface, Nanopure water droplets were not retained; the control group was designed without foliar exposure.

**Surfactant Foliar Exposure Assay.** Nonylphenoxy polyethoxy ethanol (Sigma Aldrich, Part No. 98379-10ML-F), xanthan gum (Sigma Aldrich, Part No. G1253-100G), and triethyl citrate (Sigma Aldrich, Part No. W308302-SAMPLE-K) were employed. Nonylphenoxy polyethoxy ethanol was used as a conventional surfactant, since it is the major (92%) component of Agral90, a commonly used nonionic wetting and spreading agent, which also contains 8% isobutanol. Compared with the conventional nonionic wetting agent, xanthan gum has higher biodegradability and is a renewable resource. The addition of triethyl citrate has been shown to improve xanthan gum wettability and moisture retention on the leaf surface.[38] The applied concentration of the SA was based on the suggested dosage from the manufacturer: 1 g/L of nonylphenoxy polyethoxy ethanol (Nonionic SA), and for the Green SA mixture, 0.5 g/L of xanthan gum and 5 g/L of triethyl citrate were adopted.

A total of 60 $\mu$L of surfactant per plant (120 $\mu$L/pot) were evenly distributed on the second and third leaf abaxial side near the stem using a micropipette (4−5 $\mu$L per drop). The selection of the leaf abaxial side was to assist the detachment of the SAs from the micropipette to the plant leaf surfaces and to ensure the consistency of exposures. At the applied concentration, no leaf burn effect was observed for either surfactant. After foliar exposure to the corresponding surfactant, plant samples were harvested on day 2, day 4, and day 8 to observe plant responses at different time intervals. Plant roots were washed under running DI water to remove adhered particles followed by immersing the entire plant in DI for 20 min and once again rinsed with running DI water.[39] This allowed removing loosely bounded particles from both plant roots and shoots. Then plants were separated into roots, stems, and leaves for freeze-drying followed by storage in a −80 °C freezer until the analysis.

**Physiological and Nutrient Data Measurements.** The freeze-dried plant tissues were weighed to evaluate the effects of foliar exposure to the different SAs on overall plant growth status. Then digestion was carried out in a heat block (SCP Science, DigiPREP) for nutrient measurement.[24,27,40] For more details, 2 mL of $HNO_3$ (Fisher Scientific, Product No. A509P212) was first added into the preweighed plant tissue digestion tubes (50 mL, SCP Science, Item No. 010-500-263) and heated for 20 min at 115 °C. Then 8 mL of $H_2O_2$ (Thermo Scientific, Product No. H325-4) was added followed by an hour digestion period at the same temperature. At the end, the content was diluted to the 50 mL scale mark for the multielement analysis. Four macronutrients (Ca, Mg, K, and P) and five micronutrients (Cu, Fe, Mo, Mn, and Zn) were analyzed using inductively coupled plasma mass spectrometry (Agilent Technology, Agilent ICP-MS 7900). All calibration curves had high linearity with $R^2 > 0.99$, and quality control standards (Agilent #5188-6564) were


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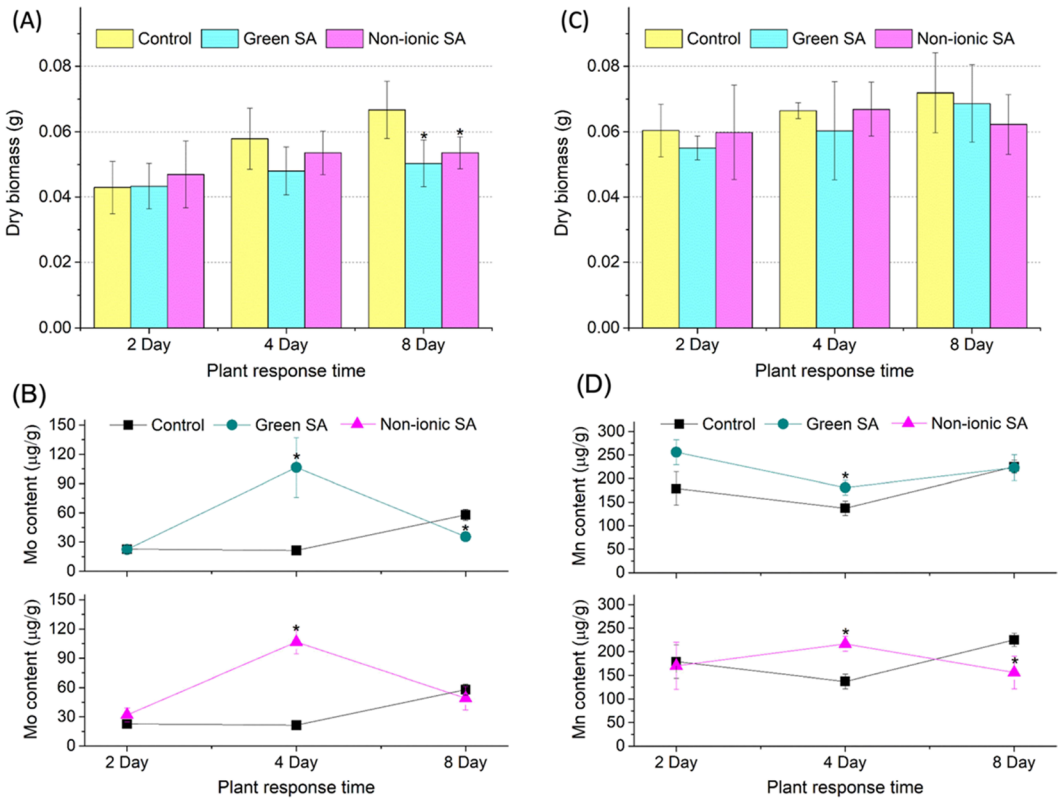


**Figure 1.** (A) Dry biomass and (B) Mo content changes with time when SAs were applied at the ET stage. (C) Dry biomass and (D) Mn content changed with time when SAs were applied at the FL stage. ET represents the ET stage (day 21) and FL stands for the flag leaf stage (day 32). Error bars indicate data standard deviation and the star symbol represents the statistical significance ($p < 0.05$).

analyzed for every 8 samples. A one-way analysis of variance (ANOVA) followed by a Tukey honestly significant difference test ($p$ value = 0.05) was performed for physiological and nutrient data analyses.[24,26]

**Metabolomic Analysis.** Metabolite data were collected using an Agilent 1260 Agilent LC triple-quadrupole mass spectrometer (LC−MS/MS). Prior to LC−MS/MS analysis, the freeze-dried plant tissues were first ground with liquid nitrogen and weighed to around 20 ± 5 mg in a 1.5 mL microcentrifuge tube (Fisher Scientific, Product No. 05-408-129).[4,24,28] The extraction process was carried out in the following steps: (1) 1.2 mL of 80% methanol + 2% of formic acid was added to the microcentrifuge tube containing preweighed plant tissues; (2) sampling tubes were vortexed for 20 min followed by another 20 min for sonication; (3) sampling tubes were centrifuged at 20,000$g$ for 20 min; and (4) the supernatant was transferred into four 2 mL autosampler vails, each containing 200 $\mu$L of sample extracts. Depending on the properties of the compounds, instrument operating conditions/ parameters (e.g., solvent mobile phases, column, solvent gradient, etc.) were adjusted accordingly to optimize LC−MS/MS analyses. Detailed analytical methods for each metabolite group can be found in previous studies.[27,28] Briefly, we divided the 91 targeted metabolites into six metabolite groups: antioxidants, organic acids/ phenolics, nucleobase/side/tides, fatty acids, amino acids, and sugar/ sugar alcohols. The detailed measurement parameters (e.g., retention time, precursor ion, product ion, and linearity) are listed in the Supporting Information (Table SI-1).

Analytical results were interpreted via the Agilent MassHunter software ($\nu$.B.06.00). Then metabolite data were log-transformed and auto-scaled before statistical analyses were carried out in Metaboanalyst 5.0 (MetaboAnalyst, CA, https://www.metaboanalyst.ca/). The significantly altered metabolites under different surfactant foliar exposure conditions were identified through a one-way ANOVA test followed by Fisher's least significant difference method ($p$ value = 0.05). A principal component analysis (PCA) was conducted on the overall metabolites profile to detect the potential cluster separations between the control and experimental groups. A multiple factor/ covariate statistical analysis was used to identify the potential correlation among three variables (e.g., surfactant type, applied stage, and plant response time). The multivariant statistical analysis is very useful for multifactor studies. The effects of Nonionic SA and Green SA were also compared across different experimental conditions. Lastly, the perturbed metabolomic pathways were discerned using criteria with impact factor >0.1 and $p$ value <0.05. The identified perturbed pathways were then linked with significant or correlated metabolites to further illustrate the overall impact of surfactant foliar exposures.

## RESULTS AND DISCUSSION

**Comparison of Wheat Leaf Biomass, Nutrient Data, and the Overall Metabolomics.** Green SA and Nonionic SA foliar exposures resulted in minimal changes in physiological and mineral nutrient content in wheat leaves (Figure 1 and Tables SI-2, 3). Dry biomass remained almost constant in days 2 and 4, however, it decreased significantly (19.7−24.5%) on day 8 when SAs were applied at the ET stage (Figure 1A). However, when SAs were applied at the FL stage, regardless of the plant response time, dry biomass was very consistent (Figure 1C). When SAs were applied at the ET stage, the metal content in leaves had the most changes on day 8 for the Nonionic SA group. However, the same trend was not observed for the Green SA treatment (Table SI-2). Mo was the most often significantly changed nutrient in wheat leaves when either surfactant was applied at ET (Figure 1B). However, Mn was the only significantly changed nutrient when either surfactant was applied at FL (Figure 1D). Both metals exhibited the most alteration on day 4 (1.3−5.0 times


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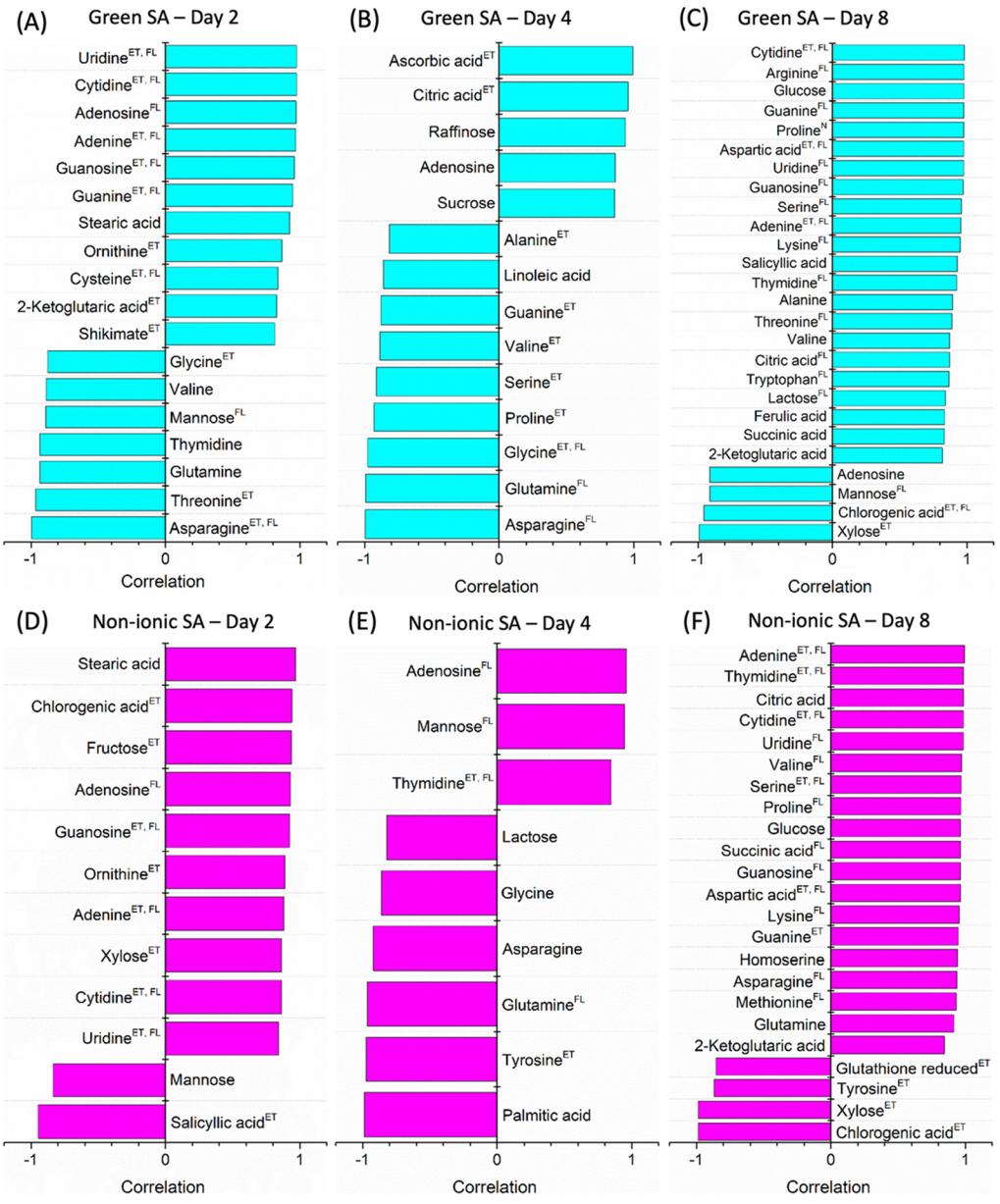


**Figure 2.** Wheat leaf metabolite levels that were highly correlated with exposure to (A–C) Green SA and (D–F) Nonionic SA, at two applied stages and different plant response times. ☐ represents Green SA and ☐ stands for Nonionic SA. Indication ET represents the metabolite level change was significant ($p < 0.05$) when the surface was applied at the ET stage and the upper letter FL at the FL collar stage. If there is no indication, the change was not statistically significant at either stage.

compared with the control) and then mostly returned to the previous levels by day 8.

For the two SAs, the applied stage and plant response times both played important roles in the overall metabolomic response (Figure SI-1 and Tables SI-4 and SI-5). When SAs were applied at ET, a nonsupervised PCA based on a total of 91 metabolites revealed that the Nonionic SA group separated well from the control for all plant response times (Figure SI-1A–C). In the case of the Green SA, the smallest metabolite profile separation was observed on day 8, compared to the separation on day 2 and day 4. This finding agreed well with the significantly altered metabolite results derived from the *t*-test, where the only altered amino acid was aspartic acid on day 8 when Green SA was applied at ET (Table SI-4). Amino acids are associated with many essential plant metabolism pathways, which also play important roles in the plant defense system to abiotic stimulus.[41] Thus, the significant decrease in the number of altered amino acid levels on day 8 indicated a fast recovery with minimal residual impact when plants were exposed to the Green SA at ET.

When the SAs were applied at FL, there was minimal change in metabolomics until day 8, when a clear separation between tested and control groups was discovered along component 1 (Figure SI-1D–F). Two times more amino acids were significantly altered on day 8 compared with earlier plant response times (Tables SI-4, SI-5). In addition, organic acids/phenolics (e.g., citric acid, malic acid, and succinic acid) only significantly responded to the SAs on day 8. The significant changes in levels of citric acid, malic acid, and succinic acid, which are intermediates in the tricarboxylic acid (TCA) cycle, clearly illustrated the perturbation of the TCA cycle. The TCA cycle is a key metabolic pathway, associated with many other


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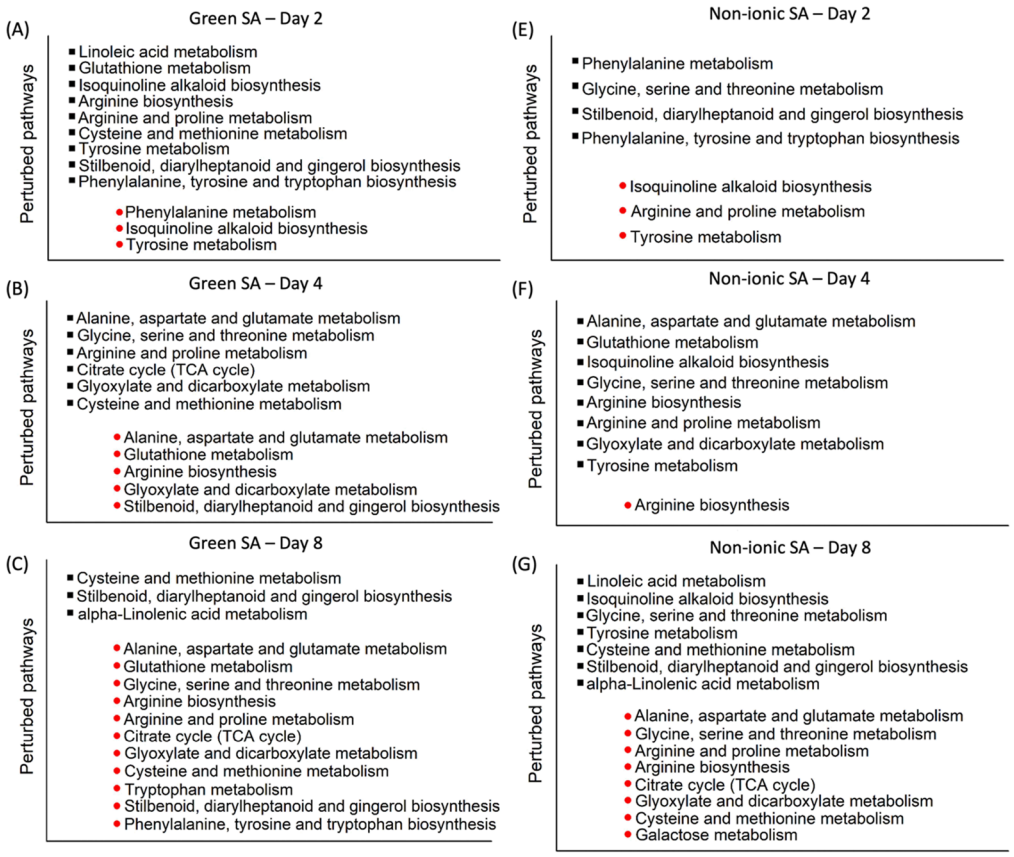


**Figure 3.** Perturbed pathways of wheat leaves that were exposed to the (A–C) Green SA and(D–F) Nonionic SA at different plant response times (days 2, 4, and 8). Square represents the surfactant was applied at the ET stage, and circle represents the surfactant was applied at the FL collar stage.

pathways, such as carbohydrates, fatty acids, and protein metabolism.[42] The significantly altered metabolite results agreed well with the PCA analysis and confirmed that when SAs were applied at FL, wheat responded to the most extent on day 8. Chlorogenic acid levels were significantly changed on day 4 and day 8 for Green SA. In contrast, no antioxidants were perturbed when Nonionic SA was applied. The significantly decreased polyphenols (e.g., chlorogenic acid) suggest impairment of the antioxidant system in wheat leaves. The hydroxyl groups on polyphenols can interact with reactive oxygen and nitrogen species due to their radical scavenging properties.[43]

**Correlation between Surfactant Application Stage and Wheat Leaf Metabolomics Response.** The stage (ET or FL) at which the SAs were applied had a greater influence on metabolite level alterations when plants were exposed to Green SA than to Nonionic SA (Figure 2 and Tables SI-6 and SI-7). Figure 2 shows the level of correlation for changes in levels of specific metabolites and exposure to either surfactant, at different stages and response times. For the period in which the plant response was monitored (from day 2 to day 8 after surfactant application), there were a total of 58 metabolite levels that were perturbed by exposure to Green SA and 44 metabolite levels altered by exposure to Nonionic SA, at the two applied stages (Figure 2). On day 2, both SAs perturbed the metabolites profile more when they were applied at ET than FL. When Green SA was applied at ET, it resulted in nine metabolic pathway perturbations on day 2, but the impact was significantly reduced by day 8, since only three pathways continued to be disturbed (Figure 3A–C). Furthermore, four of thenine9 perturbed pathways, namely, arginine and proline metabolism, arginine biosynthesis, glutathione metabolism, and phenylalanine, tyrosine and tryptophan biosynthesis, were associated with significantly changed levels of four metabolites (i.e., ornithine, 2-ketoglutaric acid, glycine, and shikimate). These perturbed pathways are related to the nitrogen metabolism. However, when Green SA was applied at FL there was no effect on these pathways (Figure 2A–C and Table SI-6). When Nonionic SA was applied at ET, only one metabolic pathway was uniquely disturbed that involved a highly correlated and significant metabolite level (chlorogenic acid), although there were several highly correlated metabolites (e.g., ornithine, salicylic acid, fructose, and xylose) that uniquely responded to ET (Figure 2D and Table SI-7). Ornithine is a nonessential amino acid involved in the urea cycle. Thus, the significant change in ornithine levels indicates the potential perturbation of the urea cycle. Salicylic acid is a plant hormone and it acts as a major signaling molecule in the plant defense system.[44] Xylose is an important structural polysaccharide (e.g., a monomer of hemicellulose) in plant cell walls and it also plays an essential role in the defense response of plants.[45] Since fructose is one of the most abundant carbohydrates in plants,[46] the dysregulation of fructose indicates the perturbation of the galactose metabolism. Thus, by the second day, wheat metabolomics were perturbed more when either surfactant was applied at ET than at FL.

By day 8, metabolic reprogramming was more extensive when SAs were applied at FL than ET, in contrast with the


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response on day 2. The majority of the metabolites with significant changes in levels were positively correlated with surfactant application. On day 8, 22 out of 26 metabolites with changed levels were positively correlated with exposure to Green SA and 19 out of 23 with exposure to Nonionic SA. These mainly contributed to the increased levels of amino acids, nucleobase/side/tide, organic acids, and sugars. Eight days after plants were exposed to Green SA at ET, no specific disturbed pathways were found that corresponded to highly correlated and significant metabolite level changes (Figure 2C and Table SI-6). However, six of 11 disturbed pathways uniquely responded when Green SA was applied at FL, which included several metabolites (e.g., arginine, citrate, threonine, and tryptophan) (Figure 3A−C). These disturbed pathways are related to the N metabolism and the TCA cycle. Some metabolites (serine, lysine, guanine, uridine, guanosine, thymidine, lactose, and mannose) were not explicitly involved in pathway perturbations, but they uniquely responded to exposure to Green SA at FL. However, under the Nonionic SA exposure condition, three unique disturbed pathways had highly correlated and significant metabolite level changes at ET (e.g., tyrosine and chlorogenic acid) and five at FL (e.g., asparagine, succinic acid, proline, and methionine.) Perturbation of pathways that resulted from exposure at FL have a more profound impact on the N metabolism in general. The increased level of succinic acid (e.g., an intermediate in the TCA cycle) demonstrated that the promotion occurred in the TCA cycle, which also generates precursors for several amino acids (e.g., asparagine, proline, and methionine). Asparagine is the main N-rich amino acid in plant leaves that is involved in N-fixation.[47] Proline is not only an important organic osmotic regulator, but it can also act as an antioxidant that regulates reactive oxygen species through synergies with antioxidant enzymes (e.g., superoxide dismutase and catalase) and peroxide metabolites (e.g., glutathione reduces and ascorbic acid) in plant cells.[48] Methionine contains sulfur which is a central intermediate in several metabolic pathways, such as cysteine and methionine metabolism. In addition, methionine is also involved in protein synthesis by initiating mRNA translation.[49] Thus, when either surfactant was applied at FL, rather than at ET, there were more profound implications for wheat metabolomics lasting for more days (e.g., day 8).

It was also interesting to note that several metabolites with negatively correlated changes in levels at an early response time switched to a positive correlation by day 8 (Table SI-6 and SI-7). For example, amino acids were mostly negatively correlated to SA application on days 2 and 4, however, nine amino acids were positively correlated on day 8 for both Green SA and Nonionic SA. In addition, concentrations of organic acid compounds also significantly increased by day 8. When Nonionic SA was applied, changes in levels of 2-ketoglutaric acid, citric acid, and succinic acid only positively correlated with applied SAs on day 8. These organic acids are intermediates from the tricarboxylic acid cycle (TCA cycle), which can also serve as precursors for amino acid biosynthesis. For example, 2-ketoglutaric acid plays an essential role in the urea cycle that includes glutamic acid, glutamine, ornithine, proline, arginine, etc.[50] In addition, citric acid is also linked with the carbohydrate metabolism through glycolysis. Upregulation of amino acids and organic acids demonstrated that when SAs were applied at FL, they not only disturbed the nitrogen metabolism but also altered the carbon metabolism by day 8.

**Magnitude of Plant Responses to Surfactant Application.** In addition to the correlation of the metabolomic response with the application of SAs at different growth stages, and subsequent response times, the magnitude of the response as measured by fold changes provides additional insights (Figure 4). Overall, the application of Green SA to wheat

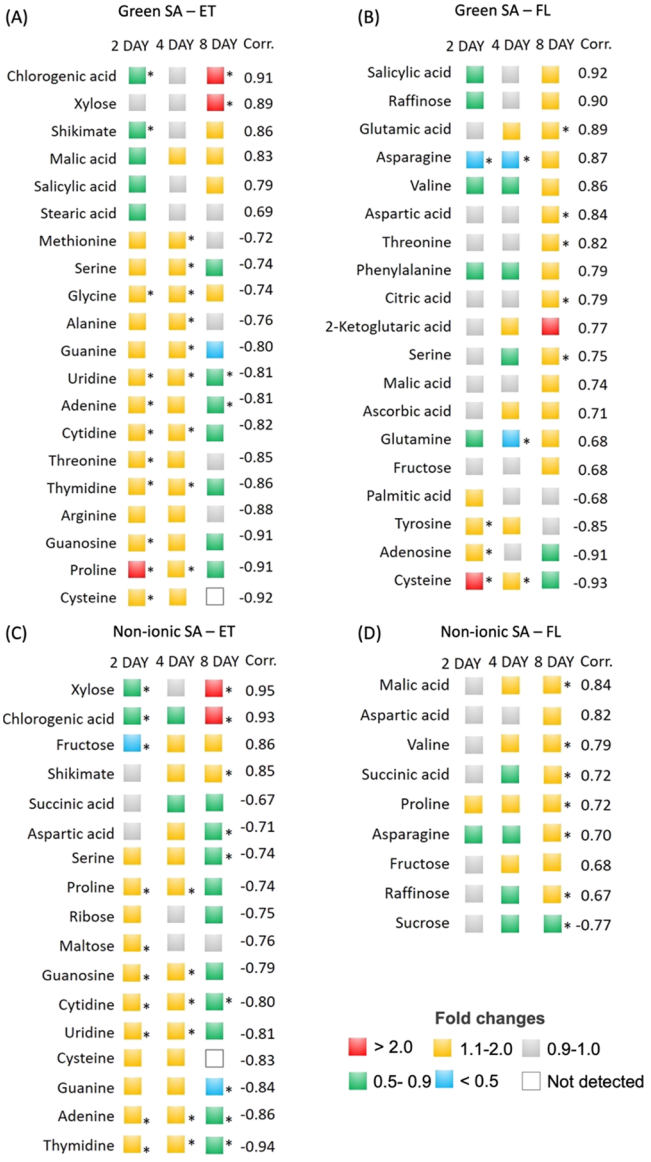


**Figure 4.** Metabolites extracted from wheat leaves that were highly correlated with the plant response time under (A and B) Green SA and (C and D) Nonionic SA exposure conditions. SAs were applied at two stages: namely, ET and the FL collar stages. Star symbol represents the statistical significance.

leaves results in a greater magnitude of metabolic reprogramming than for Nonionic SA, and it was also dependent on the applied stage and response time. The fold changes in metabolite levels were greater when either surfactant was applied at ET than at FL (Tables SI-8 and SI-9). For Green SA applied at ET, only a few metabolites had consistently increased levels with time (e.g., chlorogenic acid, xylose, shikimate, malic acid, salicylic acid, and stearic acid), while most other increased initially and then decreased with time from day 2 to day 8. For example, uridine increased initially


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(days 2 and 4), but then had levels below the control by day 8. The depletion of uridine under the Green SA exposure (at both ET and FL) indicates perturbation of the pyrimidine metabolism, which participates in broad areas of cellular metabolism, such as phosphatidylserine synthetase activity, sugar metabolism, and cell wall biochemistry (e.g., polysaccharides and glycoproteins).[51]

This initial increase in levels of certain metabolites followed by subsequent depletion was also observed under the Nonionic SA exposure. For example, cytidine, adenine, and thymidine (part of the nucleobase/side/tide group) had fold changes of 1.28−1.98 on days 2 and 4, which decreased to 0.56−0.68 by day 8. Significantly decreased levels of cytidine, adenine, and thymidine under Nonionic SA exposure demonstrated the alteration of both pyrimidine metabolism and purine metabolism.[52]

In some cases, the response was opposite when a surfactant was applied at ET rather than at FL. For example, serine and threonine had decrease levels when the Green SA was applied at ET, particularly at day 8, but their concentrations increased considerably at day 8 when applied at FL ($p < 0.05$). A similar trend was discovered for aspartic acid and proline when Nonionic SA was employed, particularly at ET. Serine could be synthesized in the amyloplast and it plays an important role in glycine synthesis through glycine hydroxyl-methyl transferase.[53] Threonine, aspartic acid, and proline all play important roles in the synthesis of defense-related metabolites,[54] so the upregulation of these metabolites could indicate the activation of the plant defense response system.[28] Overall, changes in amino acid profile may indicate a reprogramming of the nitrogen metabolism to modulate carbon and nitrogen status or to active the plant defense system upon stress.[55] Another interesting finding was that there were six nucleobases/sides/tides negatively correlated with the plant response time at ET for both SAs. However, the correlation was dramatically decreased at FL. In other words, SAs applied at ET had a greater impact on purine and pyrimidine metabolism alterations than when applied at FL.

When SAs were applied at FL, almost two times more metabolites mostly correlated positively to plant response times under the Green SA exposure than Nonionic SA (19 vs 10). The difference was mainly attributed to less altered amino acids and organic acids found in the Nonionic SA group (Tables SI-10 and SI-11). Previous studies have found that amino acids and organic acids are important metabolites to enhance plant resistance against the stimulus, and their functions include providing structure units of proteins and polypeptides, serving as precursors for other metabolites, and activating the plant defense-related system.[30,31,54] This result showed SAs applied at FL resulted in a more profound influence on wheat metabolomics for Green SA than Nonionic SA.

The Venn diagram revealed that depending on the surfactant type and applied stage, wheat pathway perturbations were quite different at the various plant response times (Figure 5). When Green SA was applied at ET, cysteine and methionine metabolism that included highly correlated metabolites (e.g., methionine and cysteine) was consistently disturbed from day 2 to day 8 (Table SI-12). As sulfur-containing amino acids, cysteine and methionine are fundamental organo-sulfur compounds that are essential for both metabolism and protein synthesis. Cysteine not only participates in the synthesis of essential biomolecules, such as antioxidants, vitamins, and co-

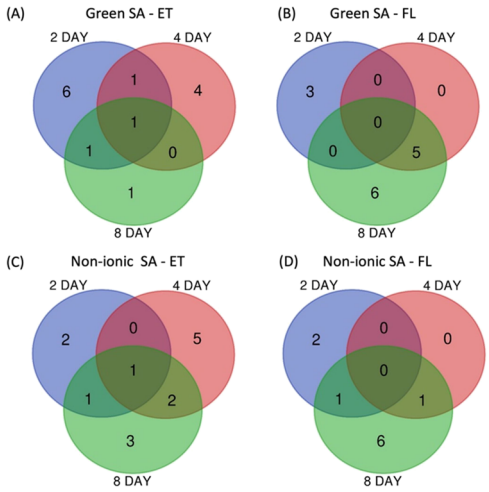


**Figure 5.** Venn diagram of perturbed metabolomic pathways with the plant response time under (A and B) Green SA and (C and D) Nonionic SA exposure conditions. SAs were applied at two stages: namely, ET and the FL collar stages.

factors, it is also important for stabilizing tertiary and quaternary protein conformation.[56] Methionine plays an important role in mRNA translation and it also serves as the precursor of *S*-adenosylmethionine for the synthesis of polyamines, vitamins, co-factors, osmoprotectants, and hormones.[56] When Green SA was applied at ET, six uniquely perturbed pathways were found on day 2, and only one additional disturbed pathway was discovered on day 8. A similar trend was not observed for the Nonionic SA. Instead, glycine, serine, and threonine metabolism (including aspartic acid) was identified as the shared perturbed pathway among plant response times (Figure 5C and Table SI-13). This perturbed pathway is related to amino acid synthesis and N metabolism. When either surfactant was applied at FL, no shared pathway perturbation was found among different plant response times. Compared with day 2 and day 4, day 8 resulted in the maximum number of disturbed pathways for both SAs. Furthermore, as one of the most important metabolic pathways, the citrate cycle (TCA cycle) was only significantly altered on day 8 (Tables SI-14 and SI-15). TCA cycle perturbation, combined with the glyoxylate and dicarboxylate metabolism, which was also disturbed on day 8 (e.g., for Nonionic SA), indicates that the carbohydrate metabolism was altered. This confirms that when SAs are applied at FL, it often results in the more metabolic reprograming by day 8.

## CONCLUSIONS

Combined with commonly studied plant physiological and metal element parameters, the current study also investigated plant responses to SAs at the molecular level. This provides additional insights into overall plant health status. Surfactant applied stages and plant response times were also included in the study to further illustrate the impact of multiple variables on wheat metabolomics. In general, few changes were observed from the physiological and nutrient data. Only when SAs were applied at ET, the dry biomass data showed a 19.7−24.5% reduction on day 8. Mo and Mn were two metal elements that exhibited the most changes in concentrations in wheat leaves when SAs were applied at either ET or FL stages.

When Green SA was applied at ET, the metabolomic profile was reprogramed the least on day 8, however, the opposite


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trend was discovered for FL. More specifically, when Green SA was applied at ET, arginine and proline metabolism, arginine biosynthesis, glutathione metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis were altered, which are metabolomic pathways that are associated with highly correlated and significant metabolites (e.g., ornithine, 2-ketoglutaric acid, glycine, and shikimate) perturbed on day 2. No specific disturbed pathways were found at the ET applied stage on day 8. In contrast, there were six uniquely perturbed pathways that responded to FL on day 8 that are linked with highly correlated and significant metabolites (e.g., arginine, citrate, threonine, and tryptophan).

When wheat leaves were exposed to the Nonionic SA at ET, the metabolite reprograming effect did not have a clear trend with plant response time. However, when it was applied at FL, day 8 also resulted in the most intense metabolic response, which was demonstrated by the well-separated metabolite profile cluster from the control in the PCA analysis, as well as increasing number of significantly altered metabolites. In particular, levels of citric acid and malic acid were only significantly altered on day 8 at FL, reflecting the perturbation of a key metabolic pathway (i.e., TCA cycle). It will be interesting to determine whether the reprogramming has long-lasting effects, beyond 8 days, in future studies. In comparing the two SAs, the Green SA resulted in more metabolic reprogramming, particularly at FL, resulting in a more profound impact on the plant defense system and nitrogen and carbon metabolism, mostly increasing the levels of perturbed metabolites by 1.1-to-2.0-fold changes. This work utilized metabolomics as a useful tool to thoroughly analyze wheat's internal responses to SAs. The results of this study provide a fundamental to surfactant-enable agriculture. Future researchers are encouraged to further explore the long-term field study, more crop species/surfactant types, a combination of multiple omics technologies, and a general better understanding of the broader environmental/ biological effects.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.2c00176.

Detailed physiological, nutrient distribution, and metabolomics data under various experimental conditions, including the control group (PDF)

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### Notes

The authors declare no competing financial interest.

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