Application timing and environmental conditions influence the effects of humic and fulvic acid on growth, yield, and cold stress response in spring wheat (*Triticum aestivum*)

By

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Abstract

Humic and fulvic acid-based biostimulants are increasingly used in agriculture to enhance plant physiological processes, improve crop performance, and, more recently, help tolerate abiotic stressors. Despite their widespread use, the optimal timing of application for greatest benefit remains poorly understood. Additionally, few studies have assessed the effects of organic acids on plants under low-temperature stress. This study examined the effects of humic and fulvic acid applications at different growth stages on physiological parameters, including photosynthetic metrics and plant nutrient levels, as well as agronomic measures in spring wheat (*Triticum aestivum* L.). A second objective was to study the effects of a humic acid seed treatment on wheat development and gene expression under low-temperature stress. The results revealed that the physiological responses to fulvic or humic acid application differed by growth stage, environmental conditions, and application timing. Fulvic acid applied during vegetative growth (wheat Zadok stage 25-37) resulted in the greatest yield increase of 9%, corresponding to 347 kg/ha of wheat, in small-plot randomized field trials. Wheat seeds treated with humic acid and subjected to low-temperature stress showed improved developmental characteristics, with cold-responsive gene expression varying by gene target and treatment. Taken together, these findings show that physiological traits and grain yield are strongly dependent on the timing of humic and fulvic acid application, with the greatest yield response occurring during early vegetative growth. Furthermore, humic acid can improve early developmental growth characteristics of wheat under low-temperature conditions, but specific gene expression should be further studied.

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Introduction and Objectives

Biostimulants are products applied to plants that enhance natural processes, such as nutrient or water uptake, and tolerance to stress (Nardi et al. 2021). Biostimulants, such as humic substances, can improve crop productivity and yield while reducing the need for conventional fertilizers, making them an appealing application option for economically important crops (Monda et al. 2021). Wheat (*Triticum aestivum*) is a commonly grown agricultural crop, with 26 million acres seeded in Canada in 2024, making it the top-grown crop in that year. In the same year, Canada produced 35 million tonnes of wheat, making it of great importance to growers and the economy. Seeded acres of wheat are projected to increase but might suffer from suboptimal yields due to unpredictable environmental conditions caused by climate change (Statistics Canada, 2024). As temperatures continue to change, action is needed to find ways to maintain yield of agriculturally important crops like wheat. Thus, agricultural products such as humic substances (HS) may provide that opportunity for growers to enhance crop productivity and yield.

Humic substances are naturally occurring compounds isolated from soil, coal, manure or other organically derived materials (Cha et al. 2020). Their structure is variable in nature, with variance resulting from where they were isolated and the fraction they consist of (García et al. 2016). Humic substances contain three main fractions: humin, humic acid (HA), and fulvic acid (FA) that vary in solubility (van Tol de Castro et al. 2021). Humin is insoluble at any pH (van Tol de Castro et al. 2021). This makes it difficult to isolate and study, which accounts for humin being uncommon in the literature (Hayes et al. 2017). Humic acid is soluble at basic pH values, while FA is soluble at all pH values (Ampong et al. 2022). The solubility of FA and HA (further referred to organic acids (OAs)), allows for easy extractability and, therefore, can be isolated and used in other forms. OAs are commonly used as a plant application, as they have known benefits to plant growth and development (Canellas et al. 2015; Ampong et al. 2022). The need for more sustainable agriculture has created a niche for OA applications on agriculturally important crops (Burgess et al. 2022).

Many studies have reported beneficial impacts of OAs on agriculturally important crops including corn (*Zea mays*) (Nunes et al. 2019), wheat (Rathor et al. 2024a, 2024b), barley (*Hordeum vulgare*) (Jarošová et al. 2016), rapeseed/canola (*Brassica napus*) (Sani 2014; Hemati et al. 2022), and potato (*Solanum tuberosum*) (Man-hong et al. 2020). In general, these studies have shown improved root and shoot development. Maximizing root area is crucial for plant growth, health, and survival, as the root system absorbs water and other essential nutrients required for the plant lifecycle (Osku et al. 2025). Maximizing shoot growth increases surface area. By increasing surface area, the amount of chlorophyll-containing tissue and area available for gas exchange increases, alongside greater total light capture. This contributes to increased photosynthetic capacity, although not necessarily to an increase in photosynthetic rate per unit leaf area (Ren et al. 2019; Yang et al. 2022). Through the optimization of photosynthesis, water, and nutrient uptake, the plant's yield can be improved (Yang et al. 2022), which is highly coveted by growers.

Essential elements are vital constituents of plant cells. These elements play a significant role in cell replication and division, macromolecule synthesis, respiration, photosynthesis, and other biological processes required for plant growth and development (Swain et al. 2023). Nutrients are often present in soils but can exist in insoluble forms and therefore are not always available for plant uptake, such as phosphorus bound in iron phosphates or calcium phosphates in acidic or alkaline soils, respectively (Zhao et al. 2023). Nutrient uptake could also be inefficient due to unfavourable soil conditions like soil composition, compaction, moisture, or pH, as these factors influence nutrient availability and root access. For example, soil composition affects nutrient retention and availability, compaction restricts root growth and reduces aeration, and soil moisture influences nutrient mobility and uptake (Griffiths and York 2020; Shaheb et al. 2021). Organic acids can chelate some essential elements, making them more available to the plant (Kontoghiorghes 2020; Rahale et al. 2021). The structures of OAs can include carboxyl, hydroxyl, carbonyl, or phenolic compounds that act as charged functional groups. These functional groups act as chelating agents, binding cationic nutrients in the soil, such as magnesium, zinc, iron, and copper. Binding to humic substances maintains these cations in soluble, plant-available forms and reduces their fixation with soil particles. In the rhizosphere, protons released by roots create acidic conditions that promote ion exchange and the subsequent release of cations from humic substances, increasing their availability for transport across root membranes (Zanin et al. 2019; Rahale et al. 2021; Rathor et al. 2024b). Nitrogen, phosphorus, sulfur, potassium, calcium, and magnesium create the essential macro-nutrients for a plant's metabolism, macromolecule synthesis, yield, and ability to cope with environmental stress (de Bang et al. 2021; Kumar et al. 2021). Eight-week-old wheat in greenhouse conditions had increased nitrogen, phosphorus, potassium, and sulphur levels when supplemented with 200, 400, and 800 kg/ha HA mixed into the soil (Rathor et al. 2024a). Similarly, nitrogen, phosphorus, potassium, calcium, and magnesium were shown to have increased in fresh leaves of wheat when the OA was applied foliarly at 45 and 60 days after sowing, with measurements taken 75 days after sowing (El-Bassiouny et al. 2014). This trend is also true for the nutrient content of wheat seed. Dincsoy and Sonmez (2019) reported an increase in phosphorus, calcium, iron, and zinc content of wheat grain when HA was mixed into the soil at 2 and 4 kg/hectare Thus, there is an opportunity to study essential elements in plant tissue following OA application. By collecting tissue at plant maturity for nutrient analysis, different OA application times can be compared to understand nutrient use and uptake better. Furthermore, the availability of essential elements within the plant are necessary for biological processes like photosynthesis (Nam et al. 2021).

Photosynthesis gives a quantitative measurement to the metabolic productivity of a plant (Calzadilla et al. 2022). Photosynthesis acts as a “base” physiological process, serving as the foundation for overall plant survival (Johnson 2016). Photosynthesis is the process by which plants convert light energy into glucose required for growth and development (Johnson 2016). Stomatal conductance, a photosynthetic parameter that controls CO_2 and O_2 exchange and water loss through stomata, influences photosynthetic rate and contributes to wheat yield by regulating CO_2 availability (Buckley 2017; Ramya et al. 2021). Other photosynthetic parameters, including chlorophyll a, can influence the light-capture potential of the photosynthetic machinery (Calzadilla et al. 2022). Chlorophyll a is an essential pigment that captures light energy during photosynthesis (Pavlovic et al. 2014). Chlorophyll fluorescence indicates the efficiency of the photosystems that utilize light energy required for photosynthesis. Effective quantum yield (ΦPSII) is a metric of chlorophyll fluorescence that compares steady state fluorescence, a measurement of the normal level of chlorophyll fluorescence under ambient conditions, to maximum fluorescence, a measure of the highest possible fluorescence the leaf can emit. This standardizes the measurement by accounting for leaf-specific properties (Chen et al. 2022; Yang et al. 2025). More chlorophyll molecules can increase the amount of light that the plant can absorb (Kalaji et al. 2016). Organic acid application has been shown to improve effective quantum yield in chrysanthemum (*Chrysanthemum morifolium)* (Fan et al. 2014) and stomatal conductance in potato (Man-hong et al. 2020) and maize (*Zea mays*) (Chen et al. 2022). Most studies examining the physiological effects of humic substances on wheat consider chlorophyll content or maximum quantum yield of photosystem II (Fv/Fm), a chlorophyll fluorescence metric for dark-adapted plants (Kraus et al. 2022; Mutlu and Tas 2022; Roy et al. 2025). Very few studies look at stomatal conductance and effective quantum yield in wheat following humic substance application. Of the limited studies that have looked at these metrics, they have shown positive results. The effective quantum yield of wheat increased relative to the control when humic acid was applied as a soil fertilizer in combination with nitrogen (Gil-Ortiz et al. 2025). Additionally, stomatal conductance increased when organic acids were applied as a humic acid soil treatment, a fulvic acid foliar spray, or both a soil treatment and a foliar spray (Mousavi et al. 2025). Though stomatal conductance and chlorophyll a play a role in photosynthetic capacity and have been shown to change in response to OA application, it is yet to be fully understood how these parameters are affected in wheat grown under field and greenhouse conditions.

In field conditions, low temperature stress negatively affects seedling emergence. Low temperature is in the range of 0-15°C (Aslam et al. 2022). Low temperature can limit emergence by preventing efficient water uptake, limiting root and shoot growth, and reducing other cellular processes such as enzymatic reactions, cell division, or electron transport (Hassan et al. 2021; Zhang et al. 2021). Therefore, mitigating low-temperature stress in crops is crucial to ensure a successful growing season. Since HA is used as an application to promote plant recovery under abiotic conditions like heat (Cha et al. 2020) or drought stress (Shen et al. 2020), HA could play a more general role in abiotic stress mitigation and provide low temperature tolerance as well (Li et al. 2023; Zhu et al. 2025). Humic acid has been studied as a technology to mitigate low-temperature stress in horticultural plants, such as zucchini (*Cucurbita pepo*) and melon (*Cucumis melo*) (Li et al. 2023; Zhu et al. 2025) and crop plants like canola (Nourgholipour et al. 2024). In general, the use of OAs to improve low-temperature tolerance is understudied in the literature, especially in wheat. Studying gene expression levels can provide insight into whether the application of HA affects the expression of genes and pathways involved in low-temperature tolerance (Upadhyay et al. 2020). The ICE-CBF-COR cascade is known to be involved in plant low-temperature regulation and comprises the inducer of CBF expression (ICE), C-repeat binding factor (CBF), and cold-regulated (*COR*) genes (Hwarari et al. 2022). When low-temperature stress is experienced, a signal cascade activates the ICE genes, leading to ICE protein upregulation of the CBF genes (Hwarari et al. 2022). When CBF transcripts are upregulated, downstream COR genes are activated (Caccialupi et al. 2023). The COR genes encode products such as dehydrins or antifreeze proteins that confer low-temperature stress tolerance by stabilizing and protecting plant membranes and proteins, or by affecting metabolism (Hwarari et al. 2022; Caccialupi et al. 2023). *TaICE87* and *TaICE41* are the wheat homologs to ICE genes in *Arabidopsis thaliana* (Badawi et al. 2008; Jung et al. 2025), *TaCBFIVd* is a wheat-specific gene that has been previously isolated and studied (Badawi et al. 2008; Jung et al. 2025), and *Wcs120*, a type of *Cor* gene, is unique to wheat

(Vítámvás et al. 2021), making them ideal gene targets to study gene expression in wheat subjected to low temperature stress.

Many studies examining the potential of organic acids as a crop application do not consider the differences resulting from varying application times. These experiments may examine the use of OAs as a seed treatment (Rathor et al. 2024b) or as a foliar spray (Mutlu and Tas 2022), but do not compare the two. Both applications have resulted in improved photosynthesis and nutrient uptake, resulting in increased biomass and yield parameters. These positive results are often not compared across application times or treatments. If timing is compared, it is indeterminate regarding which treatment yields better results (Vioratti Telles de Moura et al. 2023). Results may also vary depending on the plant, rate, application time, and if the plant is subjected to stressors such as drought, salinity, or low temperature. It is essential to determine which application timing yields the most significant improvement in plant growth and development. Determining optimal application timing would minimize cost and maximize benefits for the grower. Additionally, these studies often take place in a hydroponic medium (Ozfidan-Konakci et al. 2018) or a growth chamber (Rathor et al. 2024b), which negates the natural field conditions. It is crucial to replicate these trials in field conditions to ensure the benefits are also seen in natural conditions. To appeal to North American growers, foliar organic acids can be applied simultaneously with herbicides and fungicides, following typical agronomic practices (Iwaniuk et al. 2023).

Humic and fulvic acids can be more relevant to North American growers if they can be applied simultaneously with typical spraying practices. For growers seeking to enhance crop production, examining the benefits of different application times, such as at early vegetative and reproductive stages, can provide insight into the utility of HA and FA on crop development. This study will focus on the effects of HA and FA on the growth and development of spring wheat under greenhouse and field conditions. The OAs will be applied at different stages of the plant's lifecycle. As the plant's development and success heavily rely on early/vegetative growth (Zhao et al. 2019), I hypothesize that the seed treatment or vegetative foliar spray will have a larger effect on wheat growth and development than the reproductive foliar spray. Additionally, this study will examine the application of HA to spring wheat and how the expression of known low-temperature tolerance genes are affected under low temperature conditions. It will address the gap of comparing OA application times and examining how OAs can affect low-temperature gene expression. Because OAs are known to promote plant performance under stress, such as heat or drought, I hypothesize that OAs will have the same effect under low temperature stress and improve wheat productivity and success. Wheat provides an ideal crop for examining the application of OA in accordance with NA agricultural practices, while also offering growers a means to mitigate crop stresses, enhance plant performance, and increase overall crop yield.

Methods

Field experiment – Application timing

Field work took place at the Ian N. Morrison Research Station (Carman, Manitoba). A representative soil sample of the top 0-15 cm of soil was collected and sent to Agvise Laboratories in Northwood, ND, for testing of NPKS, pH, electrical conductivity, and % organic matter to guide fertilizer applications. Humic acid was applied as a seed treatment immediately prior to seeding, while fertilizers were applied to the soil before and during seeding as described below. Fertilizer was applied to the soil at a depth of 2-3" perpendicular to the seeding direction, following recommendations for wheat provided by Agvise. Before seeding, 16 20 0 14 (NPKS) fertilizer plus an additional 46 0 0 (NPK) fertilizer was applied to meet the target 123 kg/ha nitrogen, 22 kg/ha phosphorus, and 11 kg/ha potassium. At seeding, 11 52 0 0 (NPKS) fertilizer was applied to ensure adequate nutrient amounts and meet the target levels. *Triticum aestivum L*. var. Cardale wheat was obtained from Rob Gulden's lab at the University of Manitoba. Wheat seed was treated with 6% and 18% humic acid (HA) extracted from clay three days before seeding. In the field study, the 6% (27.5µL HA/10g seed) rate reflects the registered recommended rate for humic acid, while the 18% (82.5 µL HA/10g seed) rate was included to explore whether an increased rate could further improve plant emergence (Montra Crop Sciences, MB, Canada). Seeding was done on May 13, 2025. Each plot had a 2.5 m x 6 m seeded area. Wheat was seeded at 1" depth with 7 ½" row spacing at a target density of 400 plants/m^2. Velocity herbicide was sprayed on all plots on June 5, 2025, at 0.403L/acre.

The experimental design included four replicates of five treatments in a randomized complete block design (RCBD). The experiment included two seed treatments and three foliar spraying treatments. Wheat seeds were treated with 6% or 18% HA, or no seed treatment (Table 1). Some treatments included a 0.008% clay-extracted fulvic acid (FA) foliar spray, following the treatments listed in Table 1. SilWet L-77 (0.05% v/v) was added as a surfactant to each foliar treatment immediately prior to spraying. Fulvic acid was applied using a handheld sprayer and sprayed until the wheat was coated but not dripping. Foliar applications were sprayed at an early vegetative stage corresponding to Zadok stage

(ZS) 25-37, at a late reproductive stage, corresponding to ZS 65-70, or both (Figure 1). Foliar applications were sprayed on June 19th, 2025, and July 9th, 2025.

Table 1. Organic acid treatment applications for field trials. The vegetative foliar application corresponds to when growers typically apply herbicide. The reproductive foliar application corresponds to when growers typically apply fungicide. Both foliar applications were a 0.008% fulvic acid spray.

Treatment Number	Humic acid seed treatment	Fulvic acid foliar application	Foliar application timing
1	No seed treatment	No foliar application	N/A
2	No seed treatment	Vegetative foliar application	Zadok stage 25-37
3	No seed treatment	Reproductive foliar application	Zadok stage 65-70
4	6% humic acid	No foliar application	N/A
5	18% humic acid	Vegetative and reproductive foliar application	Zadok stage 25-37; Zadok stage 65-70

Emergence rates were taken to measure how many seedlings successfully emerged from the soil. Emergence rates were taken at 16 and 23 days after seeding. The emergence count locations were flagged to ensure the counts were repeated in the same location. A one-way analysis of variance (ANOVA) with a *post-hoc* Dunnett test was performed on emergence counts (p=0.05) for plots with a seed treatment (i.e., Treatments 4 and 5 compared to the control).

At 24 hours and seven days after each foliar application, photosynthetic measurements were taken from 20 plants per treatment. Control plots and plots receiving a foliar spray were measured. Stomatal conductance and effective quantum yield measurements were taken using a Li-Cor 600 (LI-600 Porometer/Fluorometer) according to the manufacturer's instructions (LI-COR, Lincoln, NE, USA). The Li-600 was set to a flow rate of 150 μmol/s, light-adapted, with a stability constant of within 0.005. Statistical significance was determined using a Kruskal-Wallis test and *post hoc* Dunn's test and calculated separately against the 24-hour control and the seven-day control. Following application of the vegetative-timed foliar spray, treatments #2 and #5 were compared with treatment #1 to assess photosynthetic parameters. Following the reproductive-timed foliar spray, treatments #3 and #5 were compared to treatment #1 to assess photosynthetic parameters. Percent changes were calculated separately against the 24-hour control and the seven-day control.

Macro and micronutrient content of 40 flag leaves per treatment were harvested at ten weeks after seeding (ZS 83-87) and sent to Farmer's Edge for analysis. The forty flag leaves from each treatment were pooled into one biological replicate. Nitrate, P, K, S, Ca, Mg, Cu, Fe, Mn, Zn, and B content were determined. Nitrate content was determined by combining 0.5g of dried sample with 90mL of 0.01M $CaCl_2$, filtering, and quantifying by automated colorimetric analysis following hydrazine reduction (Farmer's Edge Laboratories). The other nutrients were measured by microwave-assisted nitric acid digestion of 0.5g of dried sample, with analysis by an Inductively Coupled Plasma Optical Spectrometer (Farmer's Edge Laboratories). Percent changes were calculated against treatment #1 (control).

Plots were harvested with a small plot combine at maturity on September 10, 2025. Grain weight from each plot was converted to kg/ha to determine yield. Statistical significance for yield was determined using a one-way ANOVA (p=0.05) with a *post-hoc* Dunnett test.

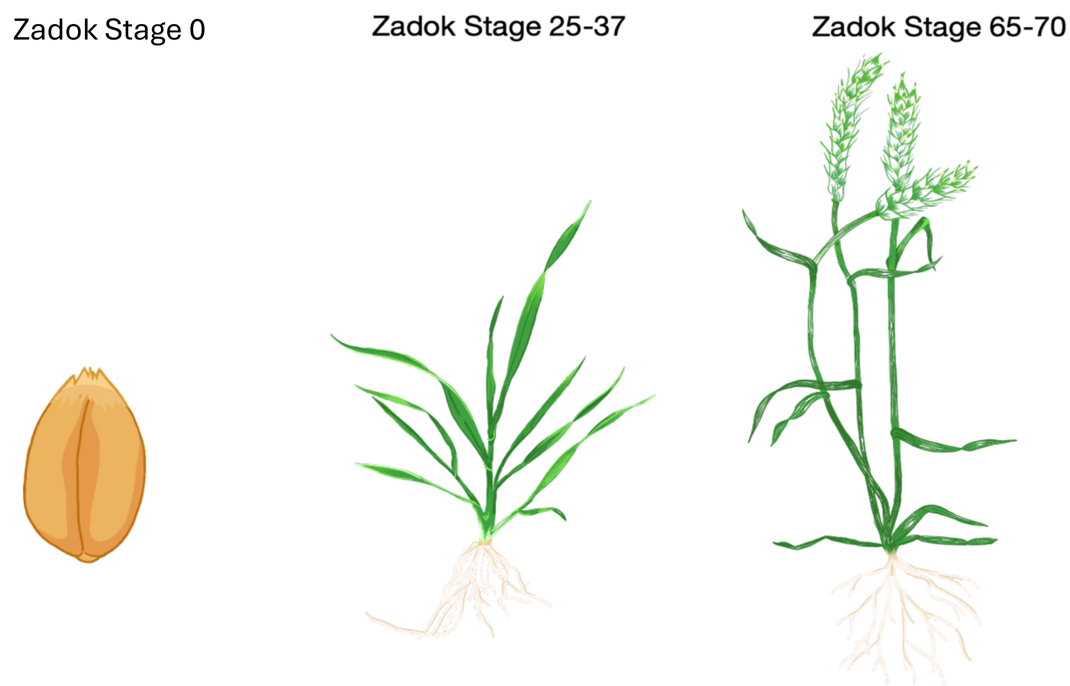

Figure 1. Staging of when organic acid treatments were applied to wheat for both greenhouse and field trials. Seed treatment was applied directly to the seed before planting. Vegetative foliar spray was applied at ZS 20-37 (tillering), depending on greenhouse or field application. Reproductive foliar spray was applied at ZS 65-70 (flowering).

Greenhouse Experiments – Application timing

A greenhouse experiment was conducted to evaluate treatment effects and physiological responses under controlled conditions. Seeds of *T. aestivum* L. var. Cardale were sown in pots containing Sunshine Mix #1 (Sungro Horticulture, Agawam, MA, USA) and supplemented with ~10 g Osmocote 14-14-14 NPK (ICL Fertilizers, Amsterdam, NL). One seed was planted at 1" depth in a 6" x 4.5" pot.

Organic acid treatments were applied as detailed in Table 2 (n = 30). Plants were arranged in an RCBD. A 6% (27.5µL undiluted HA/10g seed) HA seed treatment and 0.008% FA foliar spray was tested in this experiment (Montra Crop Sciences, MB, Canada). Since the 18% HA seed treatment did not improve emergence in the field, only the recommended 6% rate was used in the greenhouse study. SilWet L-77 (0.05% v/v) was added as a surfactant to each foliar treatment immediately prior to spraying. Wheat was sprayed until coated but not dripping. Foliar sprays were done when wheat was at ZS 20-22 and ZS 65-70 (Figure 1).

After 24 hours and seven days following foliar application, stomatal conductance and effective quantum yield measurements were taken from 20 plants per treatment. The Li-600 was used with the same settings as above. Statistical significance was determined using a Kruskal-Wallis test and *post hoc* Dunn's test.

Table 2. Organic acid treatment applications for greenhouse trials. Vegetative foliar application of fulvic acid corresponds to a herbicide timed application. Reproductive foliar application of fulvic acid corresponds to a fungicide application. Both foliar applications were a 0.008% fulvic acid spray.

Treatment Number	Humic acid Seed treatment	Fulvic acid Foliar application	Foliar application timing
1	No seed treatment	No foliar application	N/A
2	No seed treatment	Vegetative foliar application	Zadok stage 20-22
3	No seed treatment	Reproductive foliar application	Zadok stage 65-70
4	6% humic acid	No foliar application	N/A
5	6% humic acid	Vegetative and Reproductive foliar application	Zadok stage 20-22; Zadok stage 65-70

Development and yield measurements were taken from 20 plants per treatment at wheat maturity after air drying. Final shoot length was measured from the tiller conjunction point to the top of the main spike, excluding awns (Mutlu and Tas 2022). Dry shoot biomass (g) was measured by cutting the shoot at the tiller conjunction point. Number of heads was counted at maturity. Grain yield (g/plant) (El-Shabrawi et al. 2015) was measured. Statistical significance for development and yield measurements was determined using a one-way ANOVA with *post-hoc* Dunnett test, or a Kruskal-Wallis test with post-hoc Dunn test, depending on whether the data were normally distributed (Shapiro-Wilk test).

Humic acid on wheat emergence, development, and gene expression under low temperature stress

Seeds of *T. aestivum* L. var. Cardale were germinated and grown in 3”x3” pots, with three seeds per pot, containing Sunshine Mix #1 (Sungro Horticulture, Agawam, MA, USA) and supplemented with ~2.5g Osmocote 14-14-14 (ICL Fertilizers, Amsterdam, NL). Experiments were done under growth chamber conditions with a light intensity of 260µmol/m^2/s and a 16-hour photoperiod (Ganeshsan et al. 2008). The experiment was divided into three different temperature groups: 22°C, 15°C, or 10°C to simulate low temperature stress spring wheat might endure in the field (Table 3). Each temperature had three treatments: a control, a 6% HA seed treatment (27.5µL HA/10 g seed), and an 18% HA seed treatment (82.5µL HA/10 g seed). There were 33 replicates for each treatment. Plants were arranged in a RCBD.

Table 3. Growth chamber temperatures that simulate the low temperature stress wheat might face while emerging in the field.

Temperature	Condition	References
22ºC	Optimal	(Khaeim et al. 2022; Sharma et al. 2022)
15ºC	Suboptimal	(Khaeim et al. 2022)
10ºC	Low-temperature	(Khaeim et al. 2022)

Emergence rates were measured daily for a 14-day period. Wheat was considered emerged when the first leaf blade was visible above the soil surface. Percent emergence was calculated from a total of 33 plants.

As it is unlikely for wheat seedlings to experience constant temperatures through the day and into the night, different temperatures were chosen for wheat development studies with varying day/night temperatures. Seeds of *T. aestivum* L. var. Cardale wheat was germinated and grown in the same planting conditions as noted above. Experiments were done under growth chamber conditions with the light intensity of 150 $\mu mol/m^2/s$ and a 12-hour photoperiod. The experiment was separated into two temperature regimes: 20°C/15°C and 10°C/5°C day/night temperatures (Islam et al. 2021). Treatments were the same as noted above. Both temperature regimes had a five-day germination period in 20°C/15°C conditions. After the germination period, plants remained in 20°C/15°C for five days or were transferred to 10°C/5°C for five days. The experiment concluded at ten days as we were only interested in the short-term response of wheat seedlings. After the ten-day growth period, wheat growth parameters were examined to assess the effects of organic acids under low-temperature stress. The young wheat was cut at the root/shoot junction to measure root fresh weight (g). Shoots were measured (mm) from the root/shoot junction to the tallest point, and fresh weight (g) was measured. Roots were scanned using an Epson scanner and subsequently analyzed for total root length (mm), root surface area (mm^2), number of root tips and number of branching points using Rhizovision.

After measurements, leaves from at least four different plants were pooled together for a single biological replicate. RNA was isolated using the PureLink Plant RNA Reagent kit as per the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). *Triticum aestivum* primers were designed using Integrated DNA Technologies Primer Quest tool (https://www.idtdna.com/pages/tools/primerquest) and subsequently validated through the Primer BLAST tool (https://www-ncbi-nlm-nih-gov.uml.idm.oclc.org/tools/primer-blast/). Transcript abundance was measured on the Bio-Rad CFX96 Connect Real-Time system using SsoFast EvaGreen Supermix (Bio-rad Laboratories, Hercules, CA, USA) in 15µL reaction volumes. Each of the three biological replicates for the three treatments was tested in triplicate for each gene. Conditions for the qRT-PCR were as follows: 95.0°C for 30 seconds, followed by 39 cycles of 95.0° for 5 seconds and 56.0° for 5 seconds. Melt curves were performed at a range of 65-95°C with 0.5°C increments to assess non-specific amplification and primer dimers. Primer efficiencies were determined for each primer pair by analyzing the slope of a standard curve generated from a serial dilution of pooled cDNA. Amplication efficiency was calculated using the equation $(10^{(1/-S)} - 1) \times 100\%$, as reported previously (Dudziak et al. 2020). Relative transcript accumulation was calculated using the ΔΔCt method (Livak and Schmittgen 2001), relative to wheat translation elongation factor (Dudziak et al. 2020). All RT-qPCR primers used are listed in Supplementary Table S3. *Triticum aestivum* is a hexaploid species consisting of three subgenomes, each of which contains a homeologous copy of most genes (Powell et al. 2016). Genes were chosen so that expression values would represent total gene expression across all three sub genomes.

Statistical significance was calculated using a one-way ANOVA with a *post*-hoc Dunnett test (p=0.05) for root and shoot growth parameters. The ΔΔCt value was compared for treated plants to a control plant for each temperature treatment and organ system, with statistical significance being determined using a one-way ANOVA.

Results

The effects of organic acid application on spring wheat growth, development and physiology were measured under field conditions. A comparison of wheat plots at ZS 83-87 is shown in Figure 2A.

Effects of organic acid on wheat emergence, photosynthetic parameters, and yield under field conditions

Wheat seedling emergence was measured after seeding to test if a humic acid seed treatment affected wheat emergence. The 6% and 18% HA seed treatments had fewer wheat seedlings emerge compared to the control at both 16 and 23 days after planting. Although not statistically significant, emergence at 16 days after planting was reduced by about 5% for both the 6% and 18% HA seed treatments. At 23 days after planting, emergence was approximately 16% lower in the 6% and 18% HA treatments than in the control, and this difference was statistically significant (Figure 2B).

Stomatal conductance and effective quantum yield (ΦPSII) were measured with the LiCor600 at one and seven days after the vegetative (Zadok stage 25-37) and reproductive (Zadok stage 65-70) FA foliar sprays. Following the vegetative foliar spray, stomatal conductance decreased by 4-21% for the vegetative FA spray and combination treatments across one and seven days after application (DAA). The largest decrease was seen following the vegetative foliar spray at seven DAA, with the combination treatment significantly decreasing by 21% (Figure 3A). Following the reproductive foliar spray, stomatal conductance decreased at one DAA for the reproductive and combination treatments. At seven DAA, stomatal conductance decreased for the reproductive spray treatment but increased for the combination treatment, although not statistically significant (Figure 3B).

Effective quantum yield was measured to determine how efficiently photosystem II uses incoming light energy. No trend was seen in ΦPSII values across treatments or time periods. At one day after the vegetative spray, there was a 12% increase in ΦPSII for both the vegetative and combination treatments, compared to the control. At seven days after the vegetative foliar application ΦPSII decreased by 9% and 2% for the vegetative and combination treatments, respectively (Figure 3C). At seven days after the reproductive foliar spray, the ΦPSII increased or stayed the same for the reproductive spray and combination treatments (Figure 3D). No significant differences in PSII measurements were observed between treatments and their respective controls.

Nutrient analysis was done by Farmer's Edge on mature (ZS 83-87) wheat, with forty flag leaves being pooled into one biological replicate. The largest differences across treatments were seen in nitrate, calcium, copper, and manganese levels (Figure 4). Compared to the untreated control at plant maturity, nitrate levels increased by 85-106% across the 6% seed treatment, vegetative foliar spray, and reproductive foliar spray, whereas the combination treatment resulted in a 48% decrease (Figure 4A). Calcium levels increased by ~11% for the 6% HA seed treatment, reproductive foliar spray, and combination treatments compared to the control (Figure 4B). Copper levels increased in the 6% HA seed treatment and reproductive foliar spray by 24% compared to the control (Figure 4C). All treatments showed improved manganese levels, ranging from a 16%-40% increase (Figure 4D). Phosphorus, potassium, sulfur, magnesium, boron, iron, and zinc levels are shown in Table S1.

Yield (kg/ha) increased in both the vegetative and reproductive FA foliar application treatments; however, these increases were not statistically significant. The vegetative foliar application resulted in a 9% increase, from 3749 to 4096 kg/ha, compared with the control, while the reproductive foliar application increased yield to 3838 kg/ha (Figure 2C).

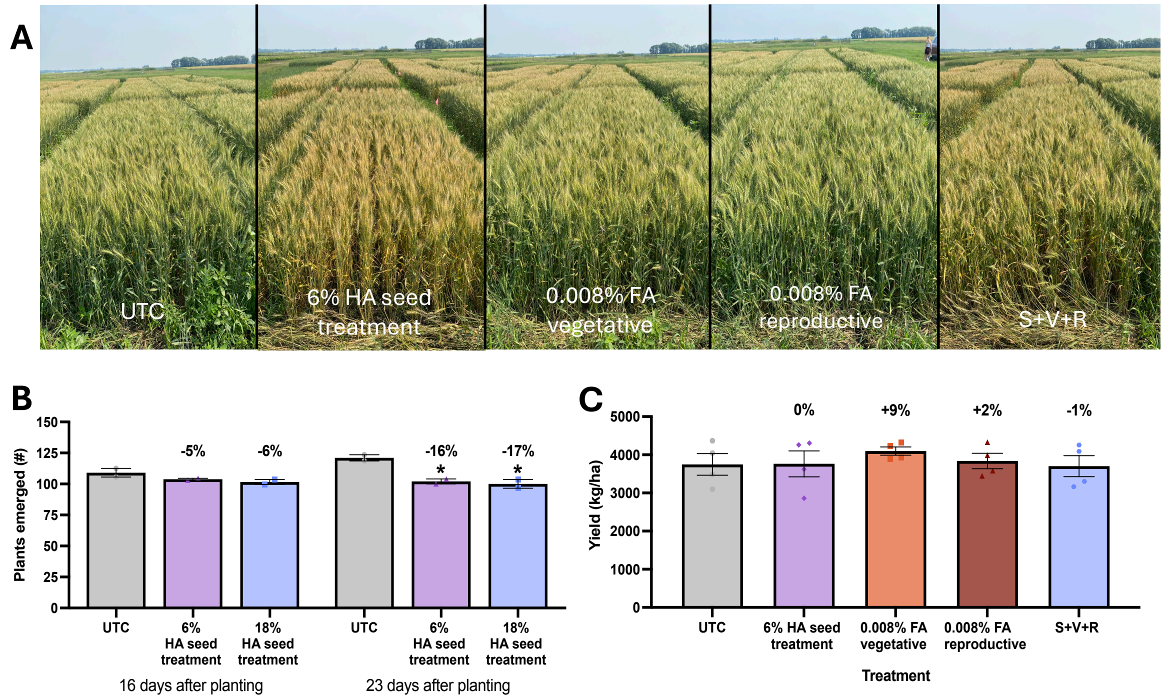

Figure 2. Effect of humic and fulvic acid treatments on spring wheat emergence and yield, taken from small plot replicates. UTC represents the untreated control, 6% HA denotes a 6% humic acid seed treatment, 0.008% FA (vegetative) and 0.008% FA (reproductive) denote foliar fulvic acid applications applied at Zadok Stages 25-37 and 65-70, respectively, and S+V+R represents an 18% humic acid seed treatment and both foliar sprays. **(A)** Wheat development at 21 days following the reproductive foliar application. **(B)** Emergence at 16 and 23 days after planting for seed-treated plots. **(C)** Yield (kg/ha). Bars represent the mean ± standard error (SE); * indicates $p<0.05$.

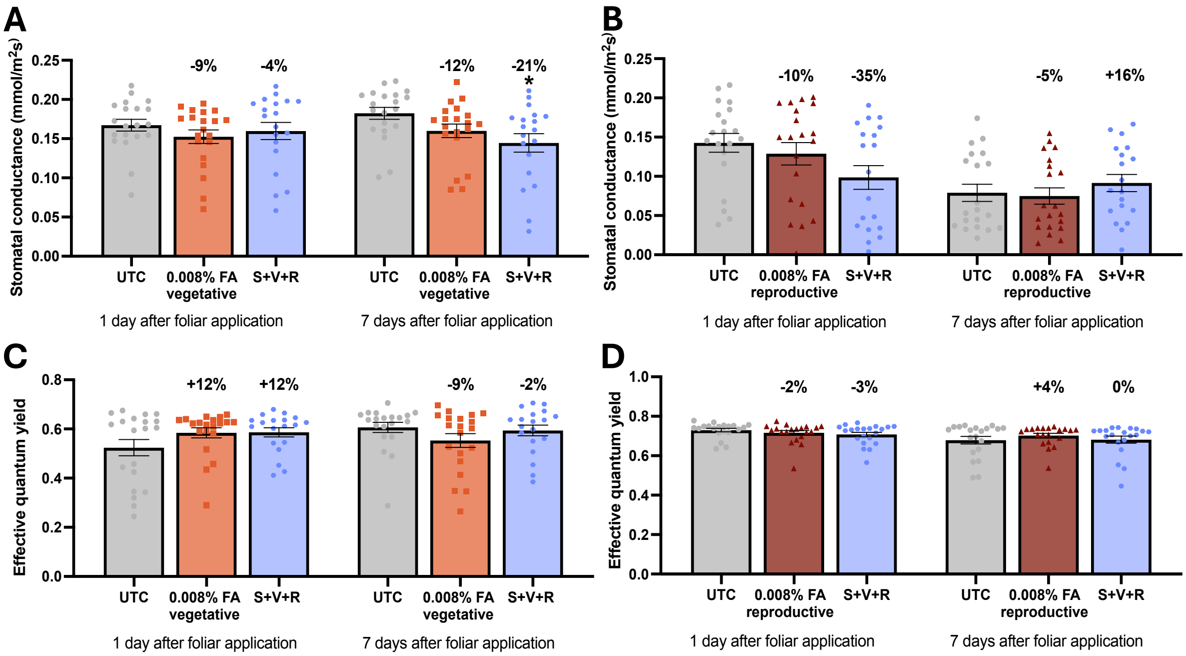

Figure 3. Effects of fulvic acid on stomatal conductance and effective quantum yield on spring wheat plants under field conditions. UTC represents the untreated control, 6% HA denotes a 6% humic acid seed treatment, 0.008% FA (vegetative) and 0.008% FA (reproductive) denote foliar fulvic acid applications applied at Zadok Stages 25-37 and 65-70, respectively, and S+V+R represents an 18% humic acid seed treatment and both foliar sprays. **(A)** Stomatal conductance (mmol/m^{2}s) measured at one and seven days following vegetative foliar application in treated plants compared to the control. **(B)** Stomatal conductance (mmol/m^{2}s) measured at one and seven days following reproductive foliar application in treated plants compared to the control. **(C)** Effective quantum yield measured at one and seven days following the vegetative foliar application in treated plants compared to the control. **(D)** Effective quantum yield measured at one and seven days following the reproductive foliar application in treated plants compared to the control. All measurements were taken from 20 plants per treatment. Bars represent the mean ± SE; * indicates $p<0.05$.

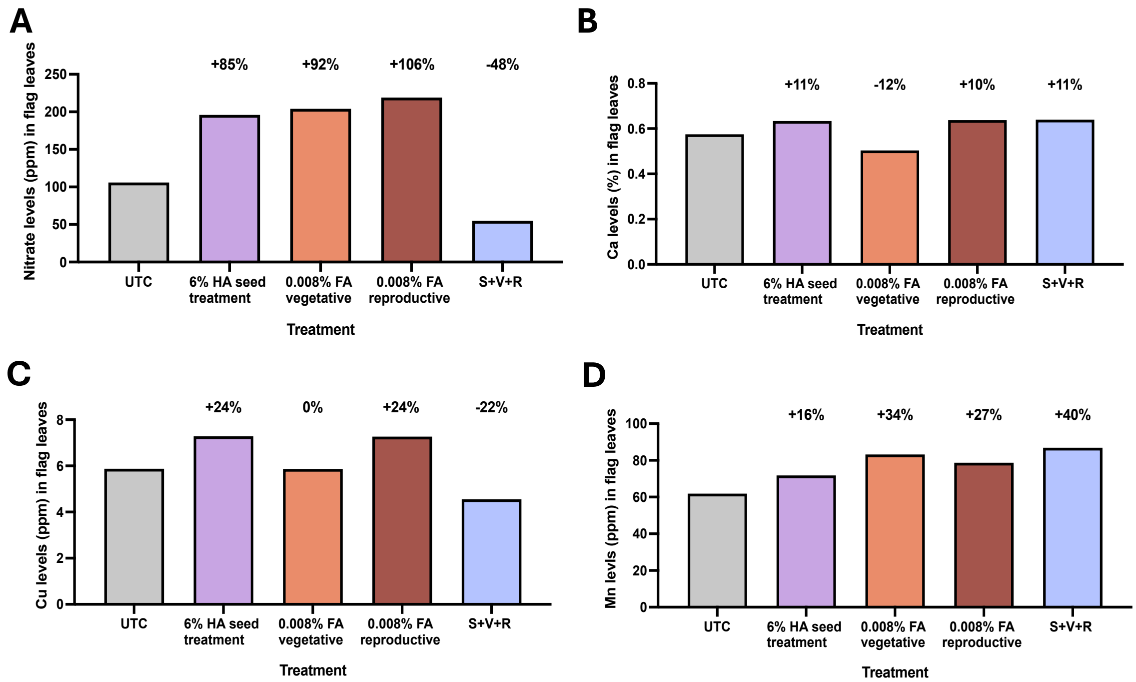

Figure 4. Effect of organic acid treatment on nutrient levels in mature spring wheat (ZS 83-87) flag leaves. Forty flag leaves were pooled into one biological replicate. **(A)** Nitrate levels (ppm). **(B)** Calcium levels (%). **(C)** Copper levels (ppm). **(D)** Manganese levels (ppm).

Effects of organic acid treatment on wheat photosynthetic, developmental, and yield parameters under greenhouse conditions

Stomatal conductance and ΦPSII were measured one and seven days after the fulvic acid foliar sprays done at vegetative (Zadok stage 25-37) and reproductive (Zadok stage 65-70) growth stages. Following the vegetative foliar application at one DAA, stomatal conductance significantly increased by 40% in the combination treatment compared to the untreated control. Stomatal conductance significantly decreased in both the vegetative spray and combination treatments seven days following the application of FA (Figure 5A). Following the reproductive foliar application at one DAA, the reproductive foliar treatment decreased by 31% while the combination treatment increased by 23%. At seven days after the reproductive spray, stomatal conductance increased for the reproductive treatment and decreased for the combination treatment compared to the control. No increases or decreases in stomatal conductance were significant following the reproductive spray (Figure 5B).

One day after the vegetative foliar spray, ΦPSII decreased significantly by 4% for both the vegetative and combination treatments, while there was no change at seven DAA (Figure 5C). Following the reproductive foliar spray at seven DAA, ΦPSII of the reproductive foliar spray decreased by 3% (Figure 5D).

Wheat development measurements were taken at plant maturity. Figure 6A shows a comparison of wheat development at ZS 71, seven days following the reproductive foliar spray. Shoot length (cm) and dry shoot biomass (g) showed minimal change across treatments (Table S2). The number of heads per plant showed the clearest treatment response. Number of heads per plant increased by 10%, 14%, 14%, and 1% for the 6% HA seed treatment, vegetative foliar spray, reproductive foliar spray, and combination treatments, respectively (Figure 6B). There was no significance across developmental measurements. A 3-6% increase in seed yield was seen across all treatments compared to the control. Grain weight per plant (g) was 10.64, 11.21, 10.96, 11.23, and 11.08 grams per plant for the control, vegetative foliar application, reproductive foliar application, 6% humic acid seed treatment, and combination treatment, respectively. The vegetative foliar application and the 6% humic acid seed treatment showed the greatest increase in grain weight per plant (Figure 6C), although not statistically significant.

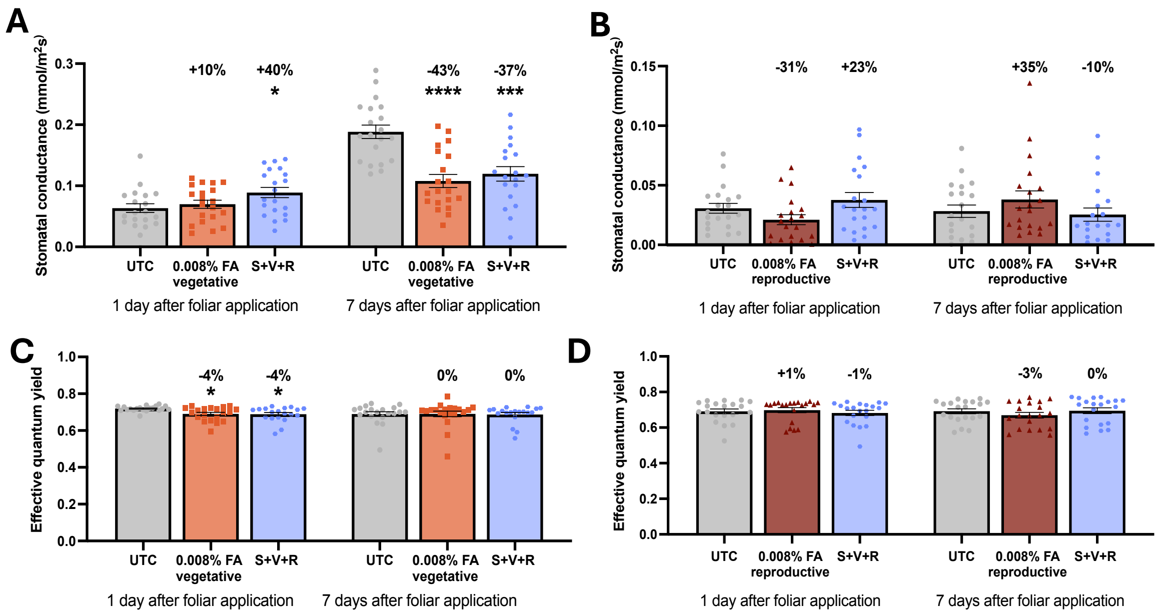

Figure 5. Effects of fulvic acid on stomatal conductance and effective quantum yield on spring wheat plants under greenhouse conditions. UTC represents the untreated control, 6% HA denotes a 6% humic acid seed treatment, 0.008% FA (vegetative) and 0.008% FA (reproductive) denote foliar fulvic acid applications applied at Zadok Stages 20-22 and 65-70, respectively, and S+V+R represents an 18% humic acid seed treatment and both foliar sprays. **(A)** Stomatal conductance (mmol/m^{2}s) measured at one and seven days following vegetative foliar application in treated plants compared to the control. **(B)** Stomatal conductance (mmol/m^{2}s) measured at one and seven days following reproductive foliar application in treated plants compared to the control. **(C)** Effective quantum yield measured at one and seven days following the vegetative foliar application in treated plants compared to the control. **(D)** Effective quantum yield measured at one and seven days following the reproductive foliar application in treated plants compared to the control. All measurements were taken from 20 plants per treatment. Bars represent the mean ± SE; * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

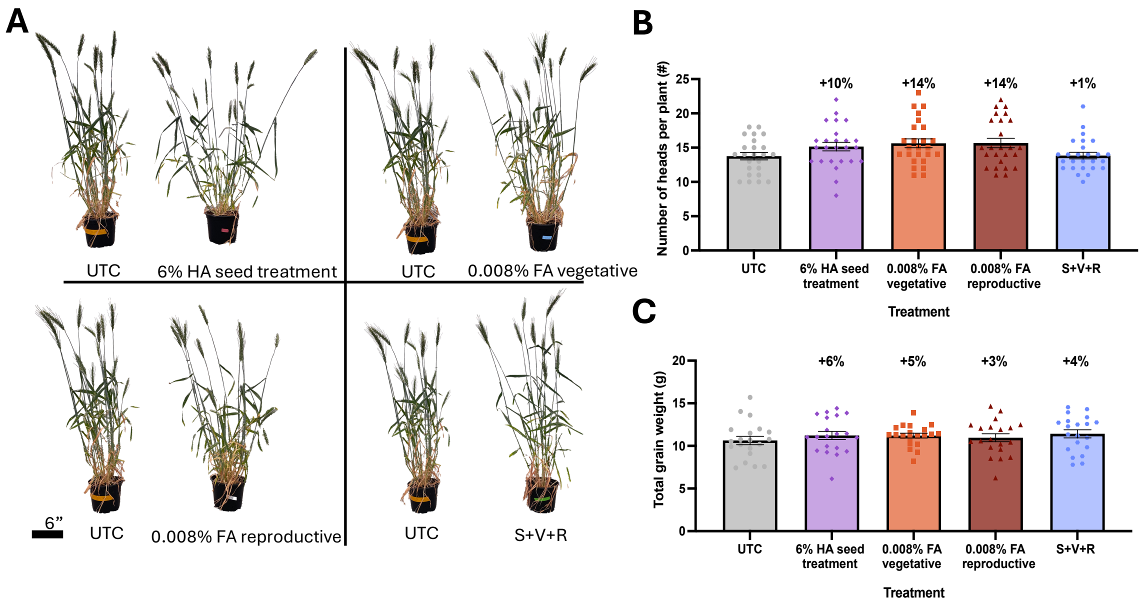

Figure 6. Effect of organic acid treatments on development and yield of spring wheat under greenhouse conditions. UTC represents the untreated control, 6% HA denotes a 6% humic acid seed treatment, 0.008% FA (vegetative) and 0.008% FA (reproductive) denote foliar fulvic acid applications applied at Zadok Stages 20-22 and 65-70, respectively, and S+V+R represents an 18% humic acid seed treatment and both foliar sprays. **(A)** Comparison of wheat development at seven days following the reproductive spray. **(B)** Number of heads per plant taken at plant maturity. **(C)** Total grain weight per plant (g). All measurements were taken from 20 plants per treatment. Bars represent the mean ± SE.

Effects of humic acid seed treatment on spring wheat emergence, development, and gene expression under low temperature conditions

Spring wheat emergence was not affected by the application of a 6% or 18% humic acid seed treatment. All plants emerged within one day of each other for each of the 22°C, 10°C, and 5°C conditions.

In a separate experiment, wheat seedlings that received a 6% or 18% humic acid seed treatment were grown under 20ºC/15ºC day/night conditions or 10ºC/5ºC day/night conditions. Figure 7A presents a comparison of ten-day-old wheat seedlings grown under 20ºC/15ºC day/night conditions following control, 6% HA and 18% HA seed treatments. Considering the 20ºC/15ºC day/night conditions, the 6% HA seed treatment resulted in a 10% increase in shoot mass (g) (Figure 7B) and a 6% increase in shoot length (mm) (Figure 7C). Shoot mass and length had a positive trend when comparing both seed treatments to the control. Under the same conditions, the 6% HA seed treatment resulted in statistically significant increases in all root parameters. Root mass increased by 30% (Figure 7D), total root length (mm) increased by 23% (Figure 7E), number of root tips increased by 46% (Figure 7F), number of branching points increased by 29% (Figure 7G), and root surface area increased by 22% (Figure 7H), following the 6% HA seed treatment.

Figure 8A presents a comparison of ten-day-old wheat seedlings grown under 10ºC/5ºC day/night conditions following control, 6% HA and 18% HA seed treatments. All parameters measured for both the 6% and 18% humic acid seed treatments increased compared to the control. The 6% HA seed treatment showed a 10% increase for shoot mass (Figure 8B) and a 4% increase for shoot length (Figure 8C). The 6% humic acid seed treatment resulted in a significant increase in all measured root parameters. The 6% HA seed treatment increased root mass by 27% (Figure 8D), total root length increased by

22% (Figure 8E), number of root tips by 37% (Figure 8F), number of branching points by 33% (Figure 8G), and root surface area by 25% (Figure 8H). The 18% HA seed treatment significantly increased root mass by 23% (Figure 8D), total root length increased by 16% (Figure 8E), and root surface area increased by 22% (Figure 8H).

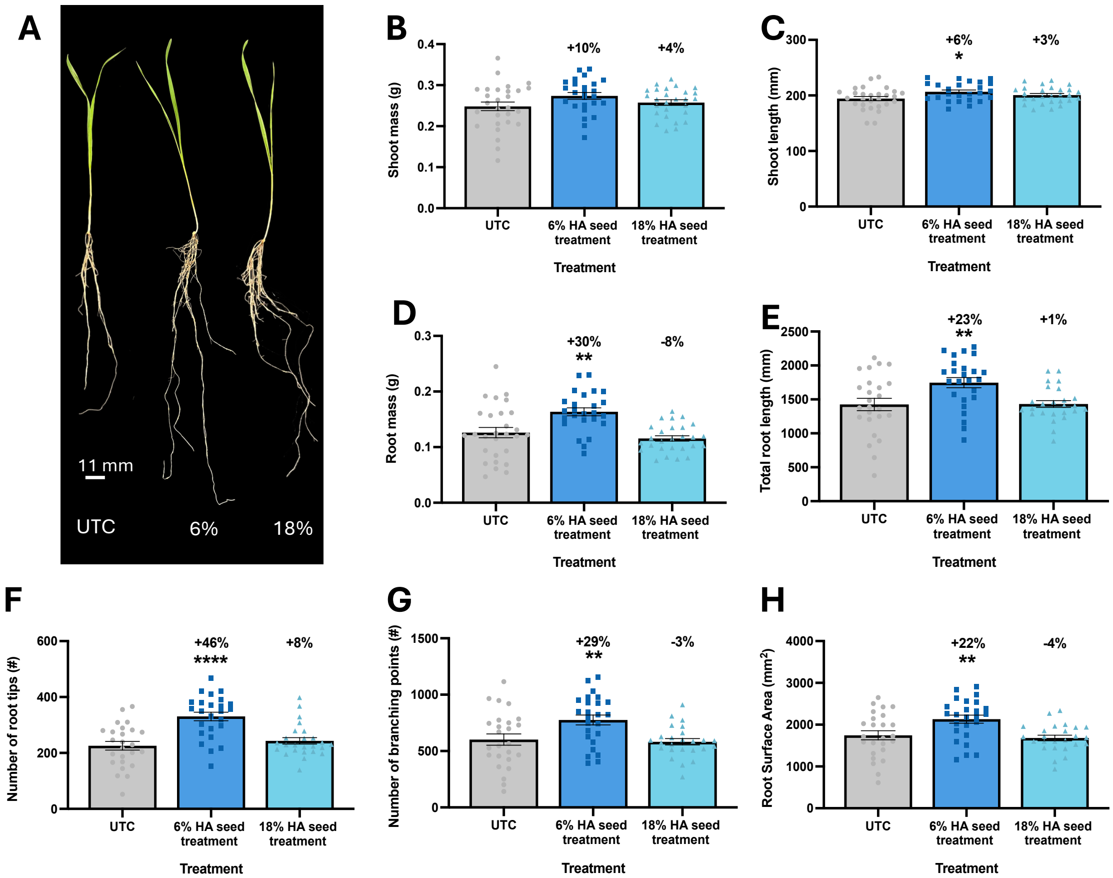

Figure 7. Effect of humic acid seed treatment on spring wheat root and shoot development under 20°C/15°C day/night conditions. **(A)** Comparison of ten-day-old wheat seedlings that received 6% and 18% humic acid seed treatment. **(B)** Fresh shoot mass (g). **(C)** Fresh shoot length (mm). **(D)** Fresh root mass (g). **(E)** Total root length (mm) as measured by Rhizovision. **(F)** Number of root tips as measured by Rhizovision. **(G)** Number of branching points as measured by Rhizovision. **(H)** Root surface area (mm^2) as measured by Rhizovision. Measurements were taken from 25 plants for all treatments. Bars represent the mean ± SE; * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

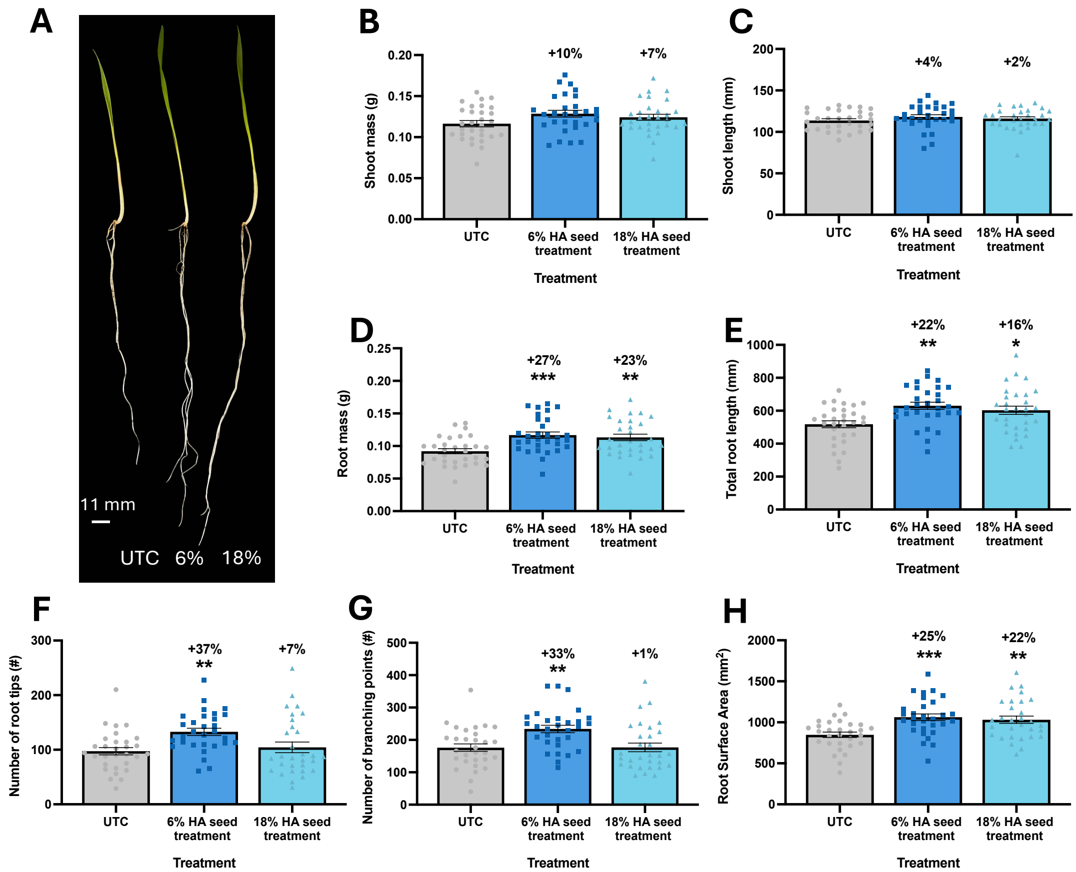

Figure 8. Effect of humic acid seed treatment on spring wheat root and shoot development grown under 10°C/5°C day/night conditions. **(A)** Comparison of ten-day-old wheat seedlings that received 6% and 18% humic acid seed treatment. **(B)** Fresh shoot mass (g). **(C)** Fresh shoot length (mm). **(D)** Fresh root mass (g). **(E)** Total root length (mm) as measured by Rhizovision. **(F)** Number of root tips as measured by Rhizovision. **(G)** Number of branching points as measured by Rhizovision. **(H)** Root surface area (mm^2) as measured by Rhizovision. Measurements were taken from 25 plants for all treatments. Bars represent the mean ± SE; * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Relative gene expression of *CBFIV* was assessed after a 6% or 18% humic acid seed treatment to determine whether expression was affected under two temperature regimes. Under the 20ºC/15ºC day/night conditions, relative expression of *CBFIV* in wheat shoots decreased for plants receiving the 6% HA seed treatment, whereas the 18% HA seed treatment increased (Figure 9A). Under the colder 10ºC/5ºC day/night conditions, both the 6% and 18% HA seed treatment increased *CBFIV* expression in the shoots (Figure 9A), although no significant differences were seen. Considering wheat roots, expression of *CBFIV* decreased significantly to 0.25 fold for the 6% HA seed treatment relative to control, but did not show a statistically significant difference for the 18% HA seed treatment under 20ºC/15ºC conditions (Figure 10B). Under 10ºC/5ºC conditions, 6% and 18% HA treatments decreased to 0.29 and 0.15 fold of the control, respectively (Figure 9B), with the 18% treatment showing a significant decrease ($p<0.0001$).

Relative gene expression of *WCS120* was studied after application of a 6% or 18% humic acid seed treatment to see if gene expression was affected under two different temperature regimes. Under the 20ºC/15ºC conditions, *WCS120* expression in wheat shoots was upregulated for plants receiving the 6% HA treatment and downregulated for plants receiving the 18% HA treatment (Figure 10A). Under 10ºC/5ºC conditions, relative expression of *WCS120* in wheat shoots did not change (Figure 10A). Considering the expression of *WCS120* in wheat roots, the 6% HA treatment did not alter expression under either 20ºC/15ºC or 10ºC/5ºC conditions (Figure 10B). Under 20ºC/15ºC conditions, root *WCS12*0 expression increased for plants receiving the 18% HA treatment (Figure 10B). No statistically significant differences were seen in *WCS120* gene expression in either tissue system or across temperature regimes.

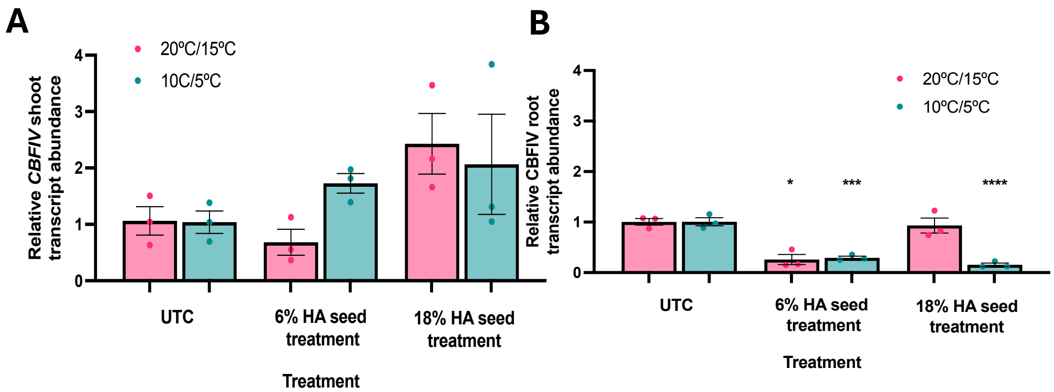

Figure 9. Relative transcript abundance of the cold-responsive gene *CBFIVd* in wheat shoots and roots under different temperatures and humic acid treatments. Treatments included an untreated control with no humic acid, 6% humic acid and 18% humic acid, with two temperature conditions per treatment. 20ºC/15ºC day/night conditions is in pink and 10ºC/5ºC day/night conditions are in green. **(A)** *CBFIVd* relative transcript abundance in wheat shoots**. (B)** *CBFIVd* relative transcript abundance in wheat roots. Bars represent the mean ± SE of n=3 biological replicates; * indicates p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

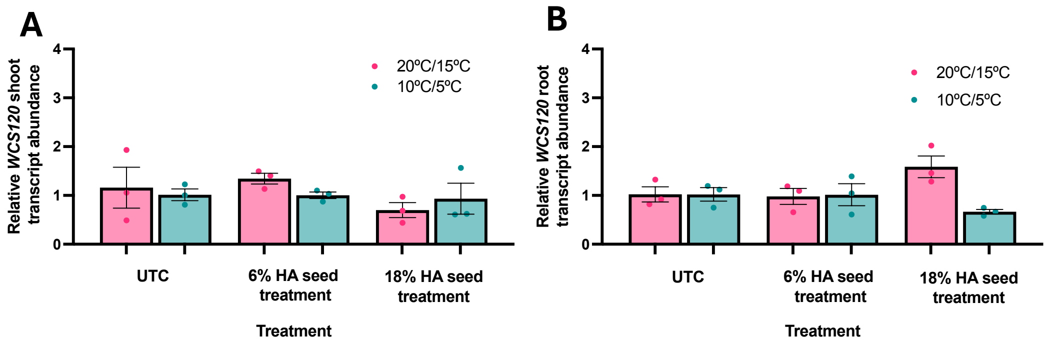

Figure 10. Relative transcript abundance of the cold-responsive gene *WCS120* in wheat shoots and roots under different temperatures and humic acid treatments. Treatments included an untreated control with no humic acid, 6% humic acid and 18% humic acid, with two temperature conditions per treatment. 20ºC/15ºC day/night conditions is in pink and 10ºC/5ºC day/night conditions are in green. **(A)** *WCS120* relative transcript abundance in wheat shoots**. (B)** *WCS120* relative transcript abundance in wheat roots. Bars represent the mean ± SE of n=3 biological replicates.

Discussion

Humic and fulvic acids are organic compounds that have been shown to positively affect physiological, growth, and developmental metrics in crop plants, including wheat (Ma et al. 2024). These studies rarely compare the effects of organic acid application timing, creating a research gap that is important to address to maximize the effects of organic acids and resulting plant productivity. This study examined how the timing of application of humic and fulvic acids affects physiological, growth, and developmental metrics of spring wheat under greenhouse and field conditions. Specifically, under field conditions, we were interested in studying wheat emergence, stomatal conductance, effective quantum yield, nutrient uptake, and seed yield. Under greenhouse conditions, we were interested in stomatal conductance, effective quantum yield, and developmental measures such as final plant height, number of wheat heads per plant, dry shoot biomass, and seed yield per plant. This study also examined the effects of a humic acid seed treatment on abiotic stress mitigation, specifically on root and shoot growth parameters and on the expression of cold tolerance genes in spring wheat under low-temperature conditions.

Wheat seedling emergence decreased in treatments receiving 6% or 18% humic acid seed treatment compared to the control. Limited field studies have looked at plant seedling emergence following a humic acid seed treatment under field conditions, making direct comparisons difficult. Germination rates and seedling vigour index are more commonly studied after seed priming or soaking with humic acid, but enhanced germination and vigour are related to improved emergence (Reed et al. 2022). Germination and vigour have been shown to improve in maize (*Zea mays*) and pumpkin (*Cucurbita pepo*) after humic acid application under salinity or cadmium stress, respectively (Asadi Aghbolaghi et al. 2022; Yang et al. 2023). Despite humic acid benefitting germination indices in other crops, emergence decreased in our trials for plots receiving a humic acid seed treatment. As humic acid was applied directly to the seed as a seed treatment, the humic acid may have limited water and oxygen uptake during germination by forming a coating on the seed surface, potentially limiting imbibition and gas exchange required for successful germination (Gorim and Asch 2017). A soil application of humic acid might provide greater benefit by improving soil properties without directly restricting water or oxygen uptake by germinating seeds, which is consistent with previous reports of improved wheat productivity following humic substance soil amendments (Tahoun et al. 2022; Rathor et al. 2024a).

Photosynthetic measurements at one and seven days after fulvic acid foliar application provide a way to measure how photosynthetic capacity changes over time, despite photosynthesis representing a short-term physiological response (Barnes et al. 2017). Previous studies have reported increases in photosynthetic parameters following organic acid application. For example, a combined humic acid and fertilizer application improved effective quantum yield in wheat by 19% (Gil-Ortiz et al. 2020). A combined humic and fulvic acid treatment increased stomatal conductance of wheat by 100% (Mousavi et al. 2025). Organic acid application has also improved stomatal conductance of maize by 97.8% and potato by up to 40% (Man-Hong et al. 2020; Chen et al. 2022). Additionally, effective quantum yield has shown to increase by 37% after organic acid application in Chrysanthemum (Fan et al. 2014). In the present study, organic acid applications did not produce clear or consistent changes in stomatal conductance across sampling time, treatment, field or greenhouse conditions (Figure 3 and 4). However, when comparing this study to others, plant-specific responses, differences in application timing, rate, and number of applications may contribute to the variability in results, as the plant response will depend on developmental stage, while rate and frequency can influence the magnitude and duration of the plant response.

Photosynthetic parameters are instantaneous and dynamic depending on weather, light availability, plant developmental stage, and potential stresses the plant is experiencing (Sharma et al. 2020). All measurements were taken within the same morning timeframe to minimize daily environmental variation. However, the sampling order was not randomized across treatments. As temperatures likely increased throughout the morning, plants measured later may have experienced slightly warmer conditions than those measured earlier. Some variation in photosynthetic parameters may therefore reflect differences in measurement timing rather than treatment effects. Future studies should randomize sampling order to minimize potential daytime temperature bias. Additionally, control plots did not receive a water-and-surfactant spray as used in the treatment plots, so potential spray-related effects cannot be fully excluded. Overall, the physiological responses of organic acid application are poorly understood and warrant further investigation. A better understanding of the physiological mechanisms would help determine the conditions and growth stages at which organic acid treatments provide the greatest agronomic benefit.

Organic acids have been shown to improve nutrient availability and increase root parameters, thereby promoting plant nutrient uptake (Zanin et al. 2019; Faria de Souza et al. 2025). The majority of previous studies have reported increases in macro- and micronutrient concentrations following humic or fulvic acid applications (Dincsoy and Sonmez 2019; Abbas et al. 2022; Barzgar et al. 2025). For example, Mousavi et al. reported that humic acid, fulvic acid, or a combination of both increased the concentration of most nutrients under both well-watered and drought conditions. In their study, combined humic and fulvic acid treatments increased calcium, manganese, and copper concentrations by 133%, 182%, and 325%, respectively (2025). Contrastingly, this study found that nutrient responses were not uniform within or across treatments. While the combined humic and fulvic acid treatment improved calcium and manganese levels by 11% and 40%, respectively, copper levels decreased. These differences may be due to variation in environmental conditions, plant developmental stage, nutrient uptake requirements, soil nutrient availability, or root uptake capacity (Weih et al. 2018). However, nutrient levels are dynamic and might not be captured by one measurement. This study was limited as tissue nutrient content was measured only at plant maturity, providing only a snapshot of final nutrient concentration. Taking these measurements at plant maturity neglects to account for stage-specific nutrient uptake. Organic acids tend to improve early-season nutrient uptake (Canellas and Olivares 2014), and by wheat maturity, many nutrients have been remobilized to grain filling and are therefore limited in the flag leaf (Jeong et al. 2016; Li et al. 2024). Studying nutrient levels earlier in the season, especially during early vegetative growth of the plant, would benefit future studies by providing a clearer picture of early-season nutrient uptake.

Nitrate levels differ from other nutrients as nitrate represents a transient inorganic nitrogen pool rather than total nitrogen (Qaderi et al. 2025). Nitrate concentration is therefore not measured as frequently in physiological studies, where nitrate uptake and assimilation are more commonly measured (Garnica et al. 2022; Han et al. 2024). Organic acids have been shown to stimulate both nitrate uptake and assimilation processes (Huertas Tavares et al. 2019; Gao et al. 2022). As nitrate concentration reflects the balance between uptake, storage, and assimilation, increased nitrate levels might represent improved nitrate uptake or reduced assimilation (Bovet et al. 2024). In the present study, increased nitrate levels for seed and foliar treatments may therefore reflect increased uptake or an accumulation of nitrate within plant tissues. Conversely, the decrease in nitrate levels observed for the combination treatment may indicate increased assimilation into organic nitrogenous compounds, reducing the overall accumulation of free nitrate in the tissues. This study measured only the nitrate content of mature leaves rather than total nitrogen, which does not account for nitrogen incorporated into amino acids, proteins, or other organic structures (Qaderi et al. 2025). Measuring total nitrogen would therefore provide a more complete picture of nitrogen composition by including both organic and inorganic forms.

Direct comparisons of organic acid application timing in relation to the wheat growth cycle remains limited, yet this factor is important for maximizing agronomic and economic benefits. Matching organic acid applications with existing agricultural practices, such as seeding, herbicide, or fungicide applications, could reduce additional input costs for growers while improving crop performance. The present study examined the overall yield response following organic acid application applied at different stages of wheat development. Despite negative effects on emergence and variable physiological responses, the fulvic acid foliar spray applied during the vegetative stage of wheat growth (ZS 20-25) produced the greatest yield response. Although not statistically significant, plots receiving the vegetative-stage fulvic acid foliar treatment increased yield by 9% compared to the control (Figure 2C). This result contradicts Alfatlawi and Alrubaiee (2020), who found that humic acid applied at the flowering stage outperformed that applied at the early stem elongation stage. This could be due to the difference in humic or fulvic acid, different soil types, environmental conditions, rate, or an interaction between these factors. Biostimulant applications in cereals have been shown to improve yield by about 10% on average, suggesting that a ~9% yield increase under field conditions may still represent a meaningful agronomic response (Li et al. 2022). Future studies could refine this application time frame by evaluating whether the 6% rate used in this study is optimal, assessing whether alternative timing within the vegetative growth stage produces similar yield increases, or evaluating whether alternative OA sources or formulations, or the addition of foliar nutrients, further improve crop productivity. Additionally, studying whether organic acid applications at similar vegetative stages across different crops would have the same yield response, providing insight as to if this response is specific to wheat or can have a broader effect.

In addition to looking at humic and fulvic acid effects under field conditions, their effects were examined under greenhouse conditions. Photosynthetic parameters measured in the greenhouse showed similar variability to those observed under field conditions, with no consistent treatment-related trends. Developmentally, the number of heads increased across all treatments. This is consistent with previous studies showing that OAs can improve the number of spikes (Alfatlawi and Alrubaiee 2020). However, this increase in spike number did not translate into a proportional increase in grain yield and was less pronounced than the yield effect observed under field conditions. This may be explained by the high number of unfilled or partially filled heads in treated plants, suggesting early processes such as tiller formation were enhanced, but did not consistently result in improved grain fill. Limited wind or physical disturbance may have lowered pollination efficiency, which could have contributed to unfilled heads. Overall, this may indicate that OA effects on final yield may be limited under optimal conditions, where resource availability reduces the need for enhanced nutrient uptake or stress mitigation.

This study also examined how 6% or 18% humic seed treatments affect wheat development and expression of cold-induced genes under low-temperature conditions. Humic acid as a mechanism for low-temperature tolerance is understudied in the literature and generally limited to horticultural crops. However, as humic acid has been shown to improve plant productivity under other abiotic stressors such as salinity, drought, and heat stress for a variety of crops (Cha et al. 2020; Chen et al. 2022; Abbas et al. 2023; Roy et al. 2025), we hypothesized that it could confer similar tolerance to low-temperature stress in wheat. In this study, root growth parameters were studied to better understand whether root architecture was affected by a humic acid seed treatment under low-temperature conditions. Wheat root architecture significantly increased across all studied parameters when applied with a 6% HA seed treatment under both 20°C/15°C and 10°C/5°C low temperature conditions (Figures 7 and 8). Under the lower-temperature regime, wheat treated with 18% HA showed increases in all root parameters, although not all increases were statistically significant. This is consistent with increases in root parameters observed in melon seedlings under low-temperature stress (Zhu et al. 2025). Improved root architecture can help with early-season plant establishment and resilience, particularly under low-temperature conditions (Wang et al. 2026). Deeper or more branched roots improve access to previously unavailable nutrients and water, while greater root surface area allows for greater nutrient and water uptake (Maqbool et al. 2022). These improvements in root architecture may support greater shoot growth and biomass accumulation, potentially contributing to increased yield. While fresh weight was used as a measure of growth in this study, it may be influenced by differences in water content and, therefore, may not fully reflect true biomass accumulation. Future studies should incorporate dry weight measurements to more accurately quantify biomass accumulation. In addition, examining the long-term response of wheat following humic acid treatment would provide a more comprehensive understanding of the treatment effect on yield under field conditions.

Gene expression analyses of the ICE-CBF-COR cold-response pathway were carried out to determine whether humic acid seed treatment influenced transcript abundance under different temperature conditions. This pathway is conserved across temperate-growing plant species and is activated in response to low-temperature stress (Aslam et al. 2022; Hwarari et al. 2022). The activation of these genes leads to the production of proteins and molecules that stabilize membranes and proteins, mitigate against oxidative damage, and maintain water balance, amongst others, which act together to improve low-temperature tolerance (Hwarari et al. 2022).

In the current study, *TaCBFIV*, a wheat-specific homolog of the CBF gene family, and *WCS120*, a COR gene encoding a dehydrin, were chosen for analysis as they are critical components in conferring cold tolerance (Caccialupi et al. 2023). These genes showed contrasting expression patterns across treatments and tissues. *TaCBFIV* expression in humic acid-treated plants increased in shoots but decreased in roots relative to the control (Figure 9). *WCS120* expression remained relatively stable across both tissue types (Figure 10). This contrasting pattern observed for *TaCBFIV* suggests tissue-specific expression, which is consistent with previous studies showing differential cold gene expression in shoots compared to roots (Guo et al. 2019; Tang et al. 2025). The reduced expression observed in roots suggests that belowground tissues may rely less on the ICE-CBF-COR pathway and instead on ICE-CBF-COR independent mechanisms to mitigate stress (Hwarari et al. 2022). These alternative mechanisms for mitigating stress may be achieved through changes in root architecture that enhance water and nutrient uptake (Canellas et al. 2024; Rathor et al. 2024b).

CBF expression can remain active throughout a stress exposure and is regulated by a number of upstream signals, including ICE transcription factors, hormonal signalling (e.g., abscisic acid) and calcium-dependent signalling (Hwarari et al. 2022; Qian et al. 2024; Li et al. 2025). The variable *TaCBFIV* expression observed in this study may reflect continued signalling under prolonged stress. It may also indicate that HA is potentially influencing gene expression by affecting stress perception or signalling pathways involved in low-temperature tolerance. However, *WCS120* did not follow the same trend as *TaCBFIV* and instead showed similar expression levels across all treatments and tissues. This suggests that *WCS120* may have peaked early in the stress response and returned to baseline prior to the day 10 sampling time (Liu et al. 2022). Thus, future studies should collect tissue samples at multiple time points following organic acid application to capture changes in transcriptome dynamics as low-temperature stress is experienced.

CBF genes are also responsive to various abiotic stresses (Kidokoro et al. 2014), suggesting that the observed changes in *TaCBFIV* expression may be a result of a general stress response. For example, under near-optimal conditions (20°C/15°C), *TaCBFIV* expression in shoots increased by 2.5-fold at the 18% HA rate, suggesting that higher HA rates may stress the plant at normal temperatures and trigger stress-related gene activity (Van Tol de Castro et al. 2021). Overall, the effects of humic acid treatments to help mitigate low-temperature stress responses in wheat appear to be tissue, temperature, and rate-dependent. The variability between *TaCBFIV* and *WCS120* expression following HA treatment suggests that modulation within the ICE-CBF-COR pathway is complex and involves other regulatory mechanisms that have yet to be studied. Further studies should examine whether additional genes involved in the low-temperature stress response, such as those encoding proline synthesis or fatty acid desaturases, are affected in humic acid-treated wheat (Hajiahmadi et al. 2020; Lv et al. 2022). Studies examining genes involved in related stress pathways would provide a more comprehensive understanding of the mechanisms by which humic acid can help to mitigate low-temperature stress in wheat (Li et al. 2023). Together, these findings provide evidence of the complexity of cold signalling in wheat in response to humic acid seed treatment.

Conclusion

Humic and fulvic acids have been widely studied for their potential to improve crop productivity, however, their efficacy varies across application rate and timing of application. This study demonstrates that the effectiveness of humic and fulvic acid applications in spring wheat is strongly dependent on application timing and environmental conditions. Specifically, foliar application of fulvic acid during early vegetative growth resulted in the greatest yield response, a factor that encapsulates all aspects of growth and development throughout the growing season, while a humic acid seed treatment enhanced root development and influenced cold-responsive processes under low-temperature conditions. These findings highlight that the timing of humic and fulvic acid application is critical for enhancing wheat performance and show the importance of targeting key developmental stages to maximize agronomic benefits. Together, these findings indicate that humic and fulvic acids can be used strategically to enhance wheat productivity and resilience, particularly when applications are aligned with critical developmental stages and environmental conditions. Overall, this work provides a foundation for optimizing the use of biostimulants in wheat production systems.

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Appendix

Table S1. Levels of macro (nitrate, phosphorus, potassium, sulfur, calcium, magnesium) and micro (boron, copper, iron, manganese, zinc) nutrient levels obtained from wheat flag leaves at Zadok Stage 83-87 under field conditions. % change was calculated against the control (UTC). Forty flag leaves were pooled into one biological replicate.

Nutrient	Expected range	UTC	6% HA seed treatment	% change (UTC to seed treatment)	vegetative FA foliar spray	% change (UTC to vegetative)	reproductive FA foliar spray	% change (UTC to reproductive)	S+V+R	% change (UTC to S+V+R)
Nitrate - N (ppm)		106	196	84.9	204	92.5	219	106.6	54.8	-48.3
Total P	0.2-0.5 (%)	0.167	0.127	-24.0	0.17	1.8	0.146	-12.6	0.167	0.0
Total K	1.5-3 (%)	2.67	2.65	-0.7	2.53	-5.2	2.63	-1.5	2.61	-2.2
Total S	0.2-0.3 (%)	0.269	0.251	-6.7	0.275	2.2	0.254	-5.6	0.325	20.8
Total Ca	0.2-1 (%)	0.575	0.634	10.3	0.504	-12.3	0.638	11.0	0.64	11.3
Total Mg	0.15-1 (%)	0.304	0.303	-0.3	0.313	3.0	0.308	1.3	0.367	20.7
Total B (ppm)	6-10 (ppm)	11.1	12.4	11.7	9.18	-17.3	11.1	0.0	11.2	0.9
Total Cu (ppm)	5-25 (ppm)	5.88	7.28	23.8	5.87	-0.2	7.27	23.6	4.56	-22.4
Total Fe (ppm)	10-150 (ppm)	130	143	10.0	121	-6.9	135	3.8	136	4.6
Total Mn (ppm)	15-100 (ppm)	61.9	71.8	16.0	83.2	34.4	78.7	27.1	86.9	40.4
Total Zn (ppm)	20-70 (ppm)	27.7	17.7	-36.1	21.5	-22.4	28.7	3.6	15.1	-45.5

Table S2. Developmental parameters (mean ± SE) of mature wheat under greenhouse conditions after organic acid application.

	Control	6% HA seed treatment	Vegetative FA foliar spray	Reproductive FA foliar spray	S+V+R
Shoot length (cm)	67.75 (0.95)	69.61 (0.96)	69.64 (0.76)	68.46 (0.72)	69.73 (0.98)
Dry shoot biomass (g)	32.12 (0.98)	31.81 (0.89)	32.37 (0.90)	31.8 (0.94)	29.44 (0.66)

Table S3. Sequences of primers used in qRT-PCR.

Gene name	GenBank accession number	Forward primer (5'-3')	Reverse primer (5'-3')
TEF-1	M90077.2	CTTTGCTACTTACCCACCTCTT	TTCTTCTCCACACCCTTGATG
TaCBFIVd	EF028783	ATTTGGGAAGAAGCAGAGTAGG	AAACCGGGAACTCGACAAATA
WCS120	M93342.2	CCGCTACCCTTGCAGAATAATA	TCTCTCCTCCAACGACCAA