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The varying promotion effects of fulvic acid with different molecular weights on the enhancement of grain yield and quality of winter wheat

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Abstract: This study aims to verify the application effect of fluvic acid (FA) with different molecular weights (MW) on the growth and quality of winter wheat. FA extracted from lignite was divided into 3 MWs (W1 ≤ 3 000 D, 3 000 D < W2 ≤ 10 000 D, and W3 > 10 000 D) by dialysis, and its structure was analysed. Three application rates were set for each MW FA in the pot experiment, which was 10, 25 and 50 mg/kg in soil, respectively, and water was the control. There were significant interactive effects of MW and application rates of FAs. Compared with the control, all the FA treatments significantly increased grain yields, nitrogen uptake efficiencies, grain iron concentration, and soil available nitrogen concentration. Heatmap analysis revealed that the W1C2 (10 mg/kg W1) treatment had the most significant impacts for all analysed indexes, whereas W3C3 (50 mg/kg W3) showed the weakest impacts. The results showed that at a low application rate (10 mg/kg in soil), the promotion effects of the three MW FAs were similar. W1 showed the most significant promotion effects, which was attributed to the combined effects of its lower MW and functional group characteristics.

Keywords: biostimulant; functional groups; iron concentration; soil nutrient concentration; nitrogen translocation

Based on current fertility levels and growth rates, scientists have predicted that the global population will reach 9.7 or 9.8 billion people by 2050 (Walker 2016). Rapid population growth and changes in dietary structure, consumption, and income growth trends will bring severe challenges in meeting food demand. It has been reported that world food production will need to increase by 70% to 100% to meet population growth needs (Tian et al. 2021). Applying mineral fertilisers has been an essential contribution to increasing global food production for a long time. However, it cannot be ignored that unreasonable application has caused a series of environmental problems (Chen et al. 2014), which has prompted people to look for green, environmentally friendly, and efficient fertiliser additives to improve

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crop yield and quality, reduce the number of mineral fertilisers, and achieve green agricultural production.

Biostimulants are natural preparations that have attracted much interest recently due to their role in improving plant growth and yield (Drobek et al. 2019). There are many biostimulants, among which humic substances (HS) have been widely used due to their large reserves and wide distribution. Fulvic acid (FA) is a component of HS, with higher content of oxygen-containing functional groups and physiological activity than that of humic acid (HA). FA can be directly dissolved in water, which is more conducive to plant absorption by and utilisation. It has been reported to enhance crop resistance to biotic and abiotic stresses and improve crop yield and quality. Moreover, FA also shows advantages in improving fertiliser use efficiency and soil physical and chemical properties (Calvo et al. 2014). The application effect of FA is remarkable in the planting and cultivating of wheat, rapeseed, corn, and other crops (Anjum et al. 2011, Braziene et al. 2021).

Due to its diverse sources and the complexity of its composition, there are significant differences in the molecular weights (MWs) and functional groups of FAs, which can lead to different agronomic effects (Baigorri et al. 2009). Although FA is a component of HS with a lower MW, it is also a macromolecule with a broad MW distribution, ranging from several hundred to tens of thousands of Daltons (Li et al. 2008, Qin et al. 2016). Macromolecules with different MWs also have differences in their functional groups and structures that affect their biological functions (Li et al. 2016, Bai et al. 2019). However, little attention has been focused on whether there are differences in the application effects of FAs with different MWs.

This study aimed to verify the application effect of FAs with different MWs on winter wheat growth. FA extracted from lignite was divided into 3 MWs (W1 ≤ 3 000 D, 3 000 D < W2 ≤ 10 000 D, W3 > 10 000 D) by a biological semipermeable membrane, and different application rates (0, 10, 25, 50 mg/kg in soil) were conducted by pot culture. Plant biomass, nutrient uptake, grain nutrient concentrations, and soil nutrient concentrations were analysed to evaluate the effects of MW and structural differences in FA on its application effect. In this study, we attempted to further enhance the biostimulator potential of this natural fertilising agent by grading its MW. It will provide scientific and rational guidance for applying FA in agricultural production to reduce fertiliser input in green agricultural production systems and improve wheat quality.

MATERIAL AND METHODS

Preparation of FA with different MWs. Potassium FA (produced by nitric oxyhydrolysis of lignite) was purchased from Xinjiang Shuanglong humic acid Co., Ltd. (Xinjiang, China). The 732 cation exchange resin was pretreated to a strong acid with 2 mol/L HCl. The FA potassium, water, and resin were mixed and stirred at a mass ratio of 1:10:20, respectively, for 5 h to carry out ion exchange to remove potassium ions. The filtered supernatant was the FA solution (Lamar et al. 2014). The FA solution was dialysed with a 3 000 D bag until the molecular diffusion inside and outside the membrane reached equilibrium. The external solution was recorded as W1 (MW ≤ 3 000 D), the internal solution was continuously dialysed with a 10 000 D bag, the collected external solution was recorded as W2 (3 000 D < MW ≤ 10 000 D), and the internal solution was recorded as W3 (FA with MW > 10 000 D). The three different MW FA solutions were concentrated by rotary evaporation.

Elemental analysis and structural characterisation of FA. The C, H, N, and S contents of the three FAs were measured by an organic element analyser (Vario EL cube, Elementary, Farhanau, Germany), and the oxygen content was calculated by subtraction. The solid ^{13}C spectrum of FA was determined with a fully digital nuclear magnetic resonance spectrometer (model: Burker 400 M, Karlsruhe, Germany) of the Bruker company in Germany. ^{13}C spectrum information of FA was measured in the range of 0~250 ppm with a 4 mm CP/MAS broadband double resonance solid probe (Liu et al. 2019b).

Plant growth conditions and experimental design. The pot experiment was conducted at the Scientific Observation Station for Nutrition and Fertilisation of Wheat and Maize Rotation in North China of the Ministry of Agriculture (Xuchang, Henan) from October 2019 to June 2020. The tested soil had a clay loam texture of 42.34% clay and was collected from the top layer of abandoned cropland at the experimental station, which had a low nutrient content. The basic physical and chemical properties of the soil were as follows: pH, 7.80; available nitrogen (N), 30.57 mg/kg; available phosphorus (P), 3.22 mg/kg; available potassium (K), 61.43 mg/kg; and organic carbon, 7.5 g C/kg. The soil was air-dried and passed through a 5-mm mesh, and then 13.0 kg of soil was weighed and placed into each polyethylene pot (30 cm tall, 30 cm diameter).

The same N, P, and K fertilisers from urea, $(NH_4)_2HPO_4$, and KCl were applied to each pot si-

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multaneously, with application rates of N 0.15 g/kg, P 0.087 g/kg, and K 0.045 g/kg. Fifteen seeds of wheat cv. Xinhuamai 818 were planted per pot on October 13, 2019, and thinned to six seedlings after emergence. The FA solution was added on March 24, 2020. Each FA of different MW was applied at three different application rates: 10 mg/kg (C1), 25 mg/kg (C2), and 50 mg/kg (C3). The control (CK) was applied with water. There were 10 treatments in total: CK, W1C1, W1C2, W1C3, W2C1, W2C2, W2C3, W3C1, W3C2, and W3C3. Each treatment had eight biological repetitions. General management for pest control and irrigation was conducted as necessary.

Plant sampling and nutrient concentration determination. Plants were sampled at anthesis (April 16, 2020) and maturity (May 28, 2020) for biomass, nutrient content, and yield analysis. At maturity, up-ground plants were divided into grains and vegetative organs (leaf, stalk, sheath, and glume). The six wheat plants in each pot were mixed into one sample, and four biological replicates were performed for each treatment.

After the biomass analysis, plant samples were ground and digested with concentrated H_2SO_4-H_2O_2 using a digestion block system for nutrient content determination. The filtrate was used to measure the concentrations of N, P, and K. The N concentration was determined with the Kjeldahl method, phosphorus was determined by molybdenum antimony anti-colourimetry, and potassium was determined by flame spectrophotometry. Grain iron (Fe) and zinc (Zn) concentrations were determined using an atomic absorption spectrophotometer after digesting ground grain with H_2SO_4-$HClO_4$.

Soil sampling and nutrient concentration determination. At maturity, three points (between two plants) were set in each pot to take 0–20 cm of soil and then air-dried to determine nutrient content after mixing evenly. The pH value was determined by the electrode method. Available P concentration was determined by the Olsen-P method. Available K was extracted, determined by 1 mol/L NH_4OAc, and determined by flame atomic absorption spectrometry. Available N was determined by the alkali diffusion method. Soil organic matter was determined by the potassium dichromate volumetric and external heating methods. Total N was analysed by the Kjeldahl method.

Calculation of relative indexes. The calculation of the translocation amount and percentage of plant dry matter and N were referenced from Cheng et al. (2019).

$$\text{DMTA (or NTA)} = \text{DMA-A (or NA-A)} - \text{DMA-VO-M (or NA-VO-M)}$$

$$\text{DMTP (or NTP)} = \text{DMTA (or NTA)} \times 100/\text{DMA-A (or NA-A)}$$

where: DMTA – dry matter translocation amount from pre-anthesis vegetative organs to grains; DMA-A – total dry matter accumulation at anthesis; DMA-VO-M – dry matter accumulation in vegetative organs at maturity; NTA – N translocation amount from pre-anthesis vegetative organs to grains; NA-A – N accumulation at anthesis; NA-VO-M – N accumulation in vegetative organs at maturity. DMTP – percentage of dry matter translocation from pre-anthesis vegetative organs to grains; NTP – percentage of nitrogen translocation from pre-anthesis vegetative organs to grains.

The nutrient uptake efficiency of N, P, and K was calculated concerning Przulj and Momcilovic (2003) method: nutrient uptake efficiency – nutrient accumulation per pot/fertilisation amount per pot.

Statistical analysis. A two-way ANOVA was conducted to analyse the significance of differences in the main effects of FA MW, application rate (AR) and their interaction. Multiple comparisons among the mean values of the main effect or the mean values of the interaction effect were determined with a least significant difference (*LSD*) test ($P < 0.05$). All the statistical analyses were performed using SPSS 23.0 (New York, USA). Heatmap (average clustering method) and PCoA (bray weighting method) analyses were performed using the OmicStudio tools at **https://www.omicstudio.cn/tool**.

RESULTS

Chemical and spectroscopic characteristics of three FAs with different MWs. The elemental compositions and atomic ratios of W1, W2, and W3 are shown in Table 1. As the MW of the FA increases, the relative carbon (C) increases while the oxygen (O) content decreases. FA (W1 and W2) with lower MWs had higher oxygen content and O/C ratio than W3. It was suggested that W3 had more condensed

Table 1. Elemental analysis of W1, W2, and W3

Sample	N	C	H	S	O	H/C	O/C
			(%)				
W1	2.13	34.27	3.51	4.63	55.46	1.23	1.21
W2	1.96	34.33	3.44	6.15	54.12	1.20	1.18
W3	1.45	50.98	3.94	2.45	41.18	0.93	0.61

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Table 2. Distribution of different carbons in W1, W2, and W3 calculated from ^{13}C NMR spectra

Sample	Carboxylic 220–161	Aromatic 161–113	Anomeric 113–93	Carbohydrate 93–44	Alkyl 44–0	HI/HB
W1	21.31	43.05	10.64	11.93	13.07	0.78
W2	23.10	46.97	10.27	9.02	10.63	0.74
W3	20.18	49.53	10.65	8.12	11.51	0.64

The percentage peak areas of individual peaks were calculated by dividing their areas by the total spectral peak area of the sample. Except for hydrophilicity (HI)/hydrophobicity (HB), the unit is %. HI/HB = ((113–44) + (200–161))/((44–0) + (161–113))

aromatic structures than W1 and W2. The higher value of H/C observed in W1 (1.23), and W2 (1.20) than in W3 (0.93) indicated significant portions of aliphatic functional groups in smaller MW FAs.

The quantitative analyses of the three FAs by solid-state ^{13}C NMR showed that the intensities of the bands of alkyl carbon (44–0 ppm) and carbohydrate carbon (93–44 ppm) regions in W1 were much higher than those in W2 and W3. The percentage peak area of the aromatic carbon region showed a trend of W1 < W2 < W3, whereas the carbohydrate carbon region showed a trend of W1 > W2 > W3 (Table 2). The HI/HB ratios (an empirical index of hydrophilicity) of W1 and W2 were higher than those of W3, suggesting that W1 and W2 were more hydrophilic than W3. In summary, W1 has higher hydrophilicity, more carbon atoms of saturated hydrocarbons, and a lower aromaticity degree.

Effects of FA with different MWs on the grain yield of winter wheat. Two-way ANOVA revealed significant interactive effects of MW and AR of FA on grain yield, spike number, and grain number per spike of winter wheat (P < 0.01 or 0.05). In contrast, there was no significant effect of FA application on thousand kernel weight (Figure 1). FA application significantly increased grain yield by 8.82~51.36%, and W1C2 treatment reached the highest yield (Figure 1A). Like grain yield, FA application significantly increased the spike number of wheat (except for W3C3), and W1C2 reached the highest among all treatments

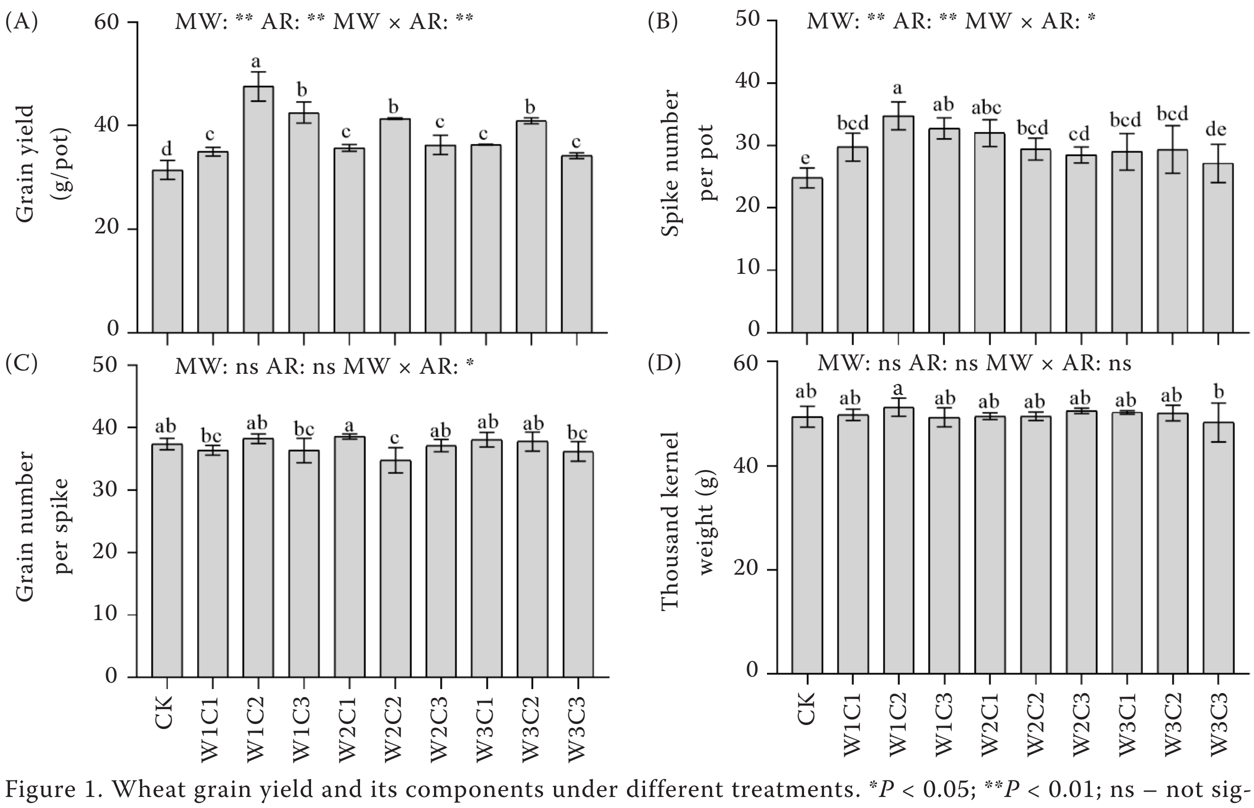

Figure 1. Wheat grain yield and its components under different treatments. *P < 0.05; **P < 0.01; ns – not significant difference; MW – molecular weight; AR – application rate

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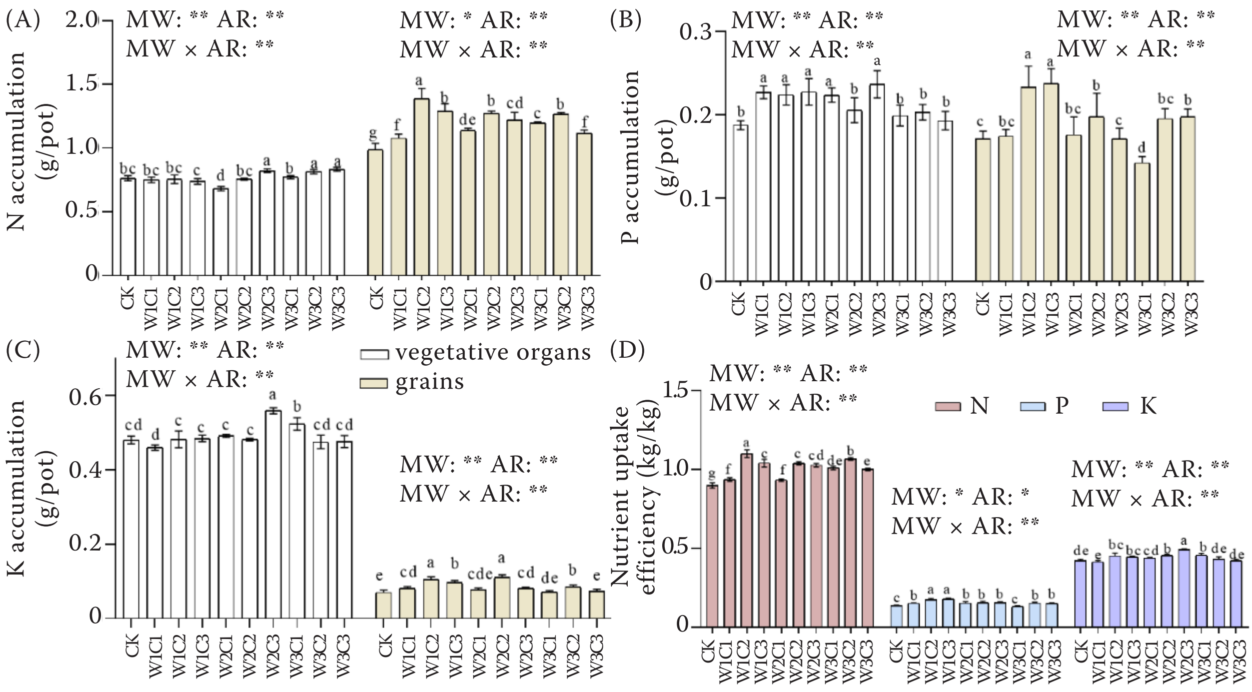

Figure 2. (A) Nitrogen (N); (B) phosphorus (P), and (C) potassium (K) accumulation amounts in vegetative organs and grains of winter wheat and (D) nutrient uptake efficiency. MW – molecular weight; AR – application rate; *P < 0.05; **P < 0.01; ns –not significant difference

(Figure 1B). FA treatments had no significant effect on the grain number per spike (except for W2C2) and thousand kernel weight of wheat (Figure 1C, D).

Effects of FA with different MWs on N, P, and K uptake in winter wheat. As revealed by two-way ANOVA, the interaction effects of MW × AR on the accumulation and uptake efficiency of N, P, and K of winter wheat were significant (P < 0.01) (Figure 2).

FA application significantly increased grain N accumulation, and the promotion effects of three FAs all reached the peaks at the C2 application rate (Figure 2A). Lower MW FA (W1) could significantly increase the grain P accumulation by 36.68% and 39.23% in W1C2 and W1C3, respectively (Figure 2B). Grain K accumulation in all W1 treatments and W2C2, W2C3, and W3C2 was significantly higher than that of CK (Figure 2C). Compared with CK, FA application significantly improved the N uptake efficiency of winter wheat by 3.83~22.39%, and the promotion effects of three FAs all reached the peaks at the C2 application rate. Except for the W3C1 treatment, FA application significantly increased the P uptake efficiency by 8.91~29.65%. Aside from the W1C1, W2C1, W3C2, and W3C3 treatments, all the other FA treatments significantly increased the K uptake efficiency compared with CK (Figure 2D).

Effects of FA with different MWs on winter wheat grain Fe and Zn concentrations. Two-way ANOVA revealed significant interaction effects of MW × AR on the concentration and accumulation of Fe and Zn in winter wheat grain (P < 0.01) (Table 3). Grain Fe concentration and accumulation of all the FA treatments were significantly higher than those of CK by 22.83~103.26% and 40.7~205.6%, respectively. Moreover, the promotion effect of W1 was much higher than those of W2 and W3, which reached the peaks at the W1C2 treatment. The promotion effect of FA application on grain Zn concentration was not as significant as that on the Fe concentration; only W1C1, W2C3, and W3C3 treatments significantly increased grain Zn concentration by 14.32, 12.47, and 12.03%, respectively, compared with CK. Grain Zn accumulation in the FA treatment was higher than in CK, except in W2C1 (Table 3).

Effects of FA with different MWs on dry matter and N translocation after anthesis. Two-way ANOVA revealed significant interaction effects of MW × AR on DMTA, DMTP, NA-A, NTA, and NTP of winter wheat (P < 0.01). Only the main effect of MW on DMA-A was significant (P < 0.05) (Table 4).

FA application significantly increased dry matter accumulation at anthesis (DMA-A), transloca-

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Table 3. The concentration and accumulation of iron (Fe) and zinc (Zn) in winter wheat grain

Treatment	Fe concentration (mg/kg)	Zn concentration (mg/kg)	Fe accumulation (mg/pot)	Zn accumulation (mg/pot)
CK	55.88 ± 7.84^{e}	24.93 ± 1.26^{b}	1.77 ± 0.34^{e}	0.79 ± 0.07^{c}
W1C1	101.61 ± 7.67^{b}	28.50 ± 2.72^{a}	3.56 ± 0.32^{c}	1.00 ± 0.04^{ab}
W1C2	113.58 ± 11.70^{a}	22.75 ± 1.06^{bc}	5.41 ± 0.48^{a}	1.08 ± 0.10^{a}
W1C3	102.35 ± 4.45^{ab}	20.79 ± 1.07^{c}	4.35 ± 0.39^{b}	0.89 ± 0.05^{bc}
W2C1	77.30 ± 7.78^{cd}	22.33 ± 1.91^{bc}	2.76 ± 0.28^{d}	0.80 ± 0.08^{c}
W2C2	83.39 ± 3.62^{c}	22.85 ± 3.45^{bc}	3.45 ± 0.17^{c}	0.94 ± 0.08^{b}
W2C3	79.71 ± 2.37^{cd}	28.04 ± 0.63^{a}	2.89 ± 0.14^{d}	1.02 ± 0.04^{ab}
W3C1	68.64 ± 5.85^{d}	24.60 ± 1.07^{b}	2.49 ± 0.23^{d}	0.89 ± 0.12^{bc}
W3C2	71.51 ± 13.81^{cd}	24.69 ± 2.12^{b}	2.93 ± 0.10^{d}	1.01 ± 0.06^{ab}
W3C3	78.47 ± 0.72^{cd}	27.93 ± 1.17^{a}	2.69 ± 0.57^{d}	0.95 ± 0.09^{b}
F-values				
MW	46.021**	2.434^{ns}	66.402**	1.918^{ns}
AR	48.916**	3.461*	86.330**	19.576**
MW × AR	6.084**	11.579**	10.709**	3.799**

Different letters after the same column of data indicate significant differences between treatments ($P < 0.05$); **$P < 0.01$; *$P < 0.05$; ns – not significant difference; MW – molecular weight; AR – application rate

tion amount (DMTA), and percentage (DMTP) by 5.4~26.6%, 12.4~75.8%, and 1.9~40.0% compared to CK, respectively. Except for W3C3, FA application remarkably enhanced the N accumulation at anthesis

Table 4. Effect of different treatments on accumulation and translocation of plant dry matter and nitrogen (N) in winter wheat

Treatment	DMA-A (g/pot)	DMTA (g/pot)	DMTP (%)	NA-A (g/pot)	NTA (g/pot)	NTP (%)
CK	92.7 ± 1.4^{e}	19.4 ± 1.7^{f}	20.9 ± 1.7^{e}	1.54 ± 0.05^{e}	0.75 ± 0.02^{f}	50.21 ± 1.93^{f}
W1C1	102.1 ± 0.8^{c}	23.4 ± 2.1^{de}	23.1 ± 2.1^{cd}	1.71 ± 0.02^{c}	0.96 ± 0.02^{cd}	56.08 ± 1.51^{c}
W1C2	117.4 ± 1.0^{a}	34.1 ± 1.2^{a}	29.0 ± 1.1^{a}	1.94 ± 0.04^{a}	1.19 ± 0.01^{a}	61.03 ± 1.52^{a}
W1C3	106.2 ± 1.7^{b}	28.1 ± 3.2^{b}	26.5 ± 2.7^{b}	1.78 ± 0.03^{b}	1.04 ± 0.01^{b}	58.61 ± 2.37^{b}
W2C1	98.2 ± 1.2^{d}	21.8 ± 0.2^{ef}	22.2 ± 0.3^{de}	1.56 ± 0.03^{de}	0.87 ± 0.01^{e}	56.08 ± 0.73^{c}
W2C2	106.9 ± 0.1^{b}	27.7 ± 1.7^{bc}	25.9 ± 1.6^{b}	1.60 ± 0.01^{d}	0.85 ± 0.01^{e}	52.85 ± 0.71^{de}
W2C3	103.6 ± 0.1^{c}	22.0 ± 1.0^{ef}	21.3 ± 1.8^{e}	1.68 ± 0.01^{c}	0.85 ± 0.02^{e}	50.90 ± 0.85^{ef}
W3C1	103.8 ± 0.1^{c}	25.3 ± 0.3^{cd}	24.4 ± 0.2^{bcd}	1.70 ± 0.02^{c}	0.93 ± 0.01^{d}	54.60 ± 0.47^{cd}
W3C2	108.1 ± 0.9^{b}	27.1 ± 1.7^{bc}	25.1 ± 1.5^{bc}	1.77 ± 0.01^{b}	0.95 ± 0.01^{c}	53.87 ± 0.73^{cd}
W3C3	97.7 ± 1.7^{d}	21.8 ± 2.1^{ef}	22.3 ± 0.9^{de}	1.55 ± 0.04^{e}	0.73 ± 0.02^{g}	46.01 ± 1.75^{g}
F-values						
MW	4.788*	19.281**	9.138**	64.653**	433.906**	57.061**
AR	0.213^{ns}	71.391**	26.820**	76.043**	441.425**	44.350**
MW × AR	0.540^{ns}	7.484**	3.966**	24.112**	135.862**	18.978**

Different letters after the same column of data show significant differences among treatments ($P \leq 0.05$). *$P < 0.05$; **$P < 0.01$; ns – not significant difference; DMA-A – dry matter accumulation at anthesis; DMT – dry matter translocation; DMTP – percentage of dry matter translocation from pre-anthesis vegetative organs to grains; NA-A – nitrogen accumulation at anthesis; NT – N translocation; NTP – percentage of N translocated from pre-anthesis vegetative organs to grains; MW – molecular weight; AR – application rate

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(NA-A), N translocation amount (NTA), and percentage (NTP) by 1.3~26.0%, 13.3~58.7% and 1.4~21.5% compared to CK, respectively. The promotion effects of the W1 application on these indexes were strong than W2 and W3 and reached the peaks at W1C2, which was significantly higher than that of the other FA treatments. These results suggest that low-MW FA is more effective at improving dry matter and nitrogen translocation from pre-anthesis vegetative to grains and that the most remarkable effects can be achieved at lower application rates.

Effects of FA with different MWs on soil nutrient concentrations at the wheat maturity stage. As revealed by two-way ANOVA, the interaction effects of MW × AR on the concentration of soil total N (TN), organic matter, available N, available P, and available K were significant ($P < 0.01$) (Table 5). FA application decreased the soil's total N concentration at maturity (except for W2C1), whereas it increased the available N concentration by 12.21~53.20% compared with CK (Table 5). Similar to the available N concentration, FA application significantly increased the available K concentration, except for W3C3. Except for W2C3 and W3C1, there was no significant difference in the soil available P concentration between the FA application and the control. All W1 application treatments and some W2 and W3 application treatments (W2C1, W2C2, and W3C1) significantly increased soil organic matter content.

Relationship between MW and application rate of FA. The cluster analysis results showed that the W1C2 treatment had the most significant impact on all indexes, followed by W1C3 (Figure 3A). W2C2 and W3C2 were preferentially clustered and further clustered with W1C3 and W1C2. Meanwhile, the results showed that W1C1, W2C1, and W3C1 had similar effects on all analysed indexes and clustered together, indicating that when the application rate was low, FAs with different MWs had a similar effect on winter wheat growth. CK and W3C3 were clustered into one class, indicating that a high application rate of high-MW FA had a weak promotion effect on the growth of winter wheat (Figure 3A). The results of the PCoA analysis also showed a similar trend. The PCoA1 axis and PCoA2 axis explained 69.91% and 13.22% of the differences in plant- and soil-related indexes under the different treatments, respectively (Figure 3B). The application of FA was mainly concentrated in I, II, and IV quadrants and the CK was in quadrant III. The W1C2 treatment and CK belong to different quadrants and do not overlap with other treatments; W1C3, W2C2, and W3C2 are like W1C2 in quadrant IV.

DISCUSSION

Many studies have proven the promotion effect of FA on plant growth and development. However, due

Table 5. Soil nutrients concentrations under different treatments at winter wheat maturity

Treatment	Total N (g/kg)	Organic matter (g/kg)	Available N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)
CK	0.70 ± 0.01^{b}	8.06 ± 0.48^{c}	35.64 ± 0.50^{e}	6.31 ± 0.22^{a}	129.98 ± 0.13^{f}
W1C1	0.60 ± 0.03^{d}	11.83 ± 0.11^{a}	52.85 ± 1.19^{ab}	5.86 ± 0.19^{abc}	142.80 ± 1.07^{ab}
W1C2	0.62 ± 0.01^{cd}	9.73 ± 0.23^{b}	43.09 ± 2.29^{d}	5.94 ± 0.65^{abc}	138.95 ± 1.89^{bcd}
W1C3	0.70 ± 0.02^{b}	9.57 ± 0.20^{b}	42.91 ± 3.77^{d}	5.98 ± 0.03^{abc}	135.34 ± 1.00^{de}
W2C1	0.74 ± 0.02^{a}	11.53 ± 0.50^{a}	43.98 ± 2.43^{d}	6.13 ± 0.22^{ab}	137.83 ± 4.49^{cde}
W2C2	0.64 ± 0.02^{c}	9.45 ± 0.53^{b}	44.63 ± 2.8^{cd}	5.85 ± 0.50^{abc}	140.49 ± 2.24^{abc}
W2C3	0.63 ± 0.01^{c}	8.29 ± 0.50^{c}	54.60 ± 2.57^{a}	5.58 ± 0.35^{bc}	138.58 ± 3.94^{bcd}
W3C1	0.70 ± 0.02^{b}	9.42 ± 0.26^{b}	39.99 ± 3.14^{de}	5.32 ± 0.36^{c}	143.73 ± 2.93^{a}
W3C2	0.68 ± 0.01^{b}	8.29 ± 0.29^{c}	48.77 ± 3.48^{bc}	6.04 ± 0.75^{ab}	137.14 ± 4.01^{cde}
W3C3	0.63 ± 0.02^{c}	8.06 ± 0.31^{c}	41.65 ± 6.56^{d}	6.23 ± 0.10^{ab}	133.74 ± 1.82^{ef}
F-values					
MW	16.735**	122.650**	30.860**	3 040.473**	95.960**
AR	1.028^{ns}	53.591**	10.983**	4.295**	5.151**
MW × AR	4.464**	19.961**	8.264**	7.062**	4.980**

Different letters after the same column of data indicate significant differences between treatments ($P < 0.05$); $^{*}P < 0.05$; $^{**}P < 0.01$; ns –not significant difference; MW – molecular weight; AR – application rate

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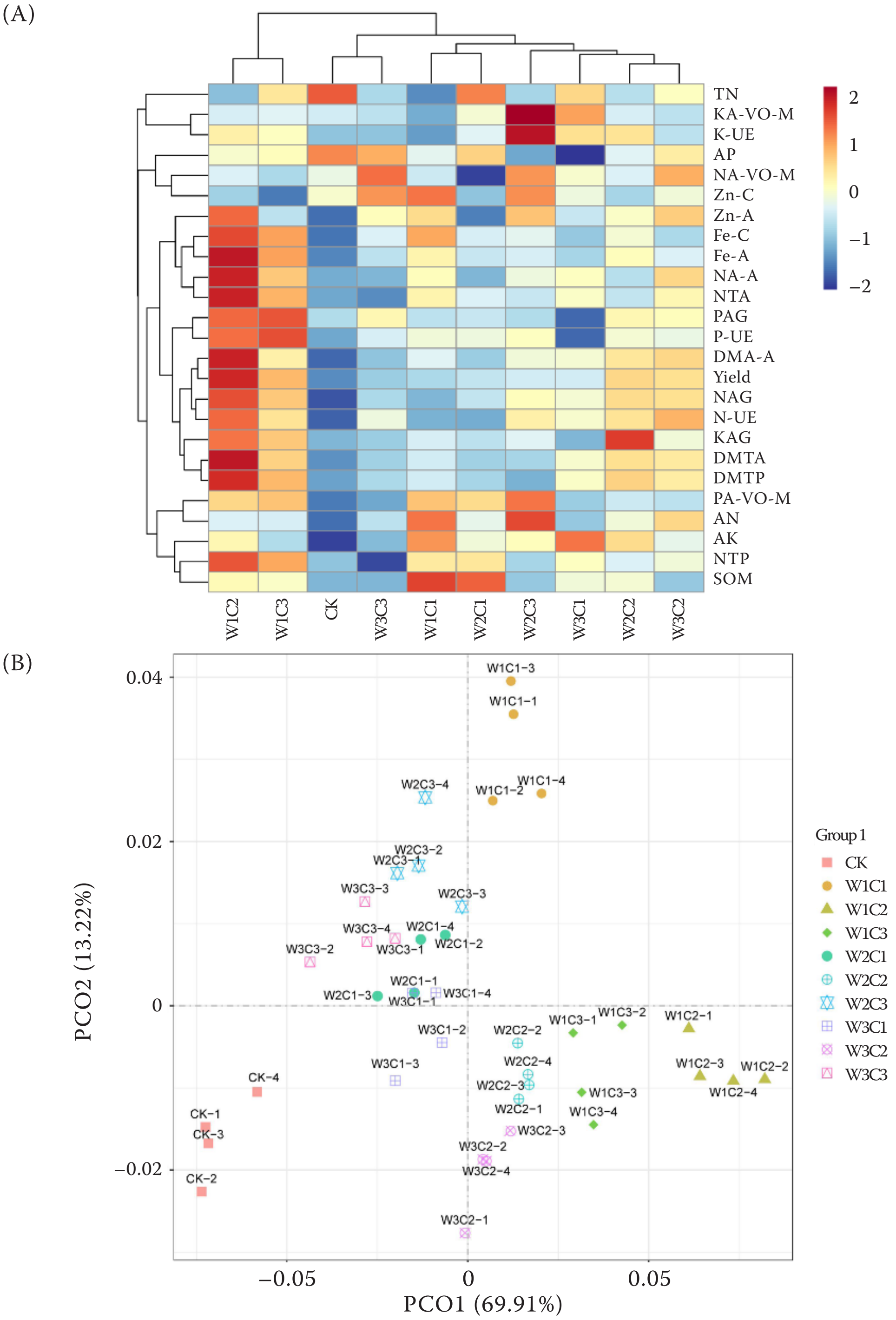

Figure 3. (A) Heatmap and (B) the principal coordinate analysis (PCoA) based on a Bray-Curtis dissimilarity matrix of the plant biomass, grain yield, nutrient uptake and soil nutrient content. DMA-A – dry matter accumulation at anthesis stage; NA-A – nitrogen (N) accumulation at anthesis stage; NA-VO-M – N accumulation in vegetative organs at maturity; PA-VO-M – phosphorus (P) accumulation in vegetable organs at maturity; KA-VO-M – potassium (K) accumulation in vegetable organs at maturity; NAG – N accumulation in grain; PAG – P accumulation in grain; KAG – K accumulation in grain; N-UE – N uptake efficiency; P-UE – P uptake efficiency; K-UE – K uptake efficiency; Fe-C – Fe concentration in grain; Zn-C – Zn concentration in grain; Fe-A – Fe accumulation in grain; Zn-A – Zn accumulation grain; DMTA – dry matter translocation amount; DMTP – dry matter translocation percentage; NTA – N translocation amount; NTP – N translocation percentage; TN – soil total N; SOM – soil organic matter; AN – available N; AP – available P; AK – available K

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to the complex molecular structure and wide range of sources of FA, its action mechanism still lacks an in-depth investigation, which hinders its practical application and promotion. It has been shown that a close relationship exists between the MW of macromolecules and their biological activities (Liu et al. 2015). It was found that different HA MWs and structures showed different promotion effects on plant growth (Quaggiotti et al. 2004, Qian et al. 2015). The present study showed that applying FA with different MWs significantly increased the plant biomass and yield of winter wheat, and the interaction effects of MW and AR were significant. In general, tiny MW FA (W1) had more excellent promotion effects on grain yield and reached the peaks at a lower application rate of 25 mg/kg soil (Figure 1). These results indicate that although FA is a small molecular component in HS, it also showed varied application effects of different MW FAs. In addition to the difference in MW, we characterised the elemental composition and structure of the three FAs and found apparent differences. W1 showed the highest carbon atoms of saturated hydrocarbons and the lowest aromaticity degree among the three FAs (Table 2). Li et al. (2016) used different sources of FAs to study their effects on biological denitrification, and the results showed that SAFA exhibited higher biological activity than the other two FAs due to its lower MW, lower aromatic structure, complexity, and higher hydrophilicity. HAs with three MWs extracted from leonhardite could significantly promote snap bean leaf and root growth. However, lower MW HAs were more able to promote root growth, and K uptake than larger MW HAs, which might be attributed to more O-alkyl and carboxyl-C groups (Qian et al. 2015). In our study, W3, having the highest MW, showed significant differences in element composition and functional groups compared with W1 and W2, which indicated that MW and molecular structure together affect the biological activity of W3. However, for the structurally similar W1 and W2, the difference in application effects between them should be mainly attributed to the difference in MW. Based on these studies, we speculated that the more excellent promotion effects of W1 on the growth and nutrient utilisation of winter wheat were due to the combined effect of its lower MW, less complex aromatic structures, higher saturated hydrocarbons, and hydrophilicity among the three FAs.

In addition to promoting plant growth, FA also provides significant advantages in promoting plant uptake of soil nutrients and improving nutrient use efficiency, especially N use efficiency. Liu et al. (2022) found that the combined application of N fertiliser and FA increased plant N and P uptake, decreased the total mineral N leaching factor, and improved nutrient utilisation efficiency in wheat. Quaggiotti et al. (2004) showed that low-MW HA induced the expression of H^+-ATPase and nitrate transporters (ZmNRT2.1 and ZmNRT1.1), thus promoting N uptake. In sandy soil, it has been reported that HS improves water retention in the root zone and increases plant-available nutrients in the soil (Selim and Mosa 2012). Sootahar et al. (2019) showed that applying liquid FA could improve the effectiveness of soil nutrients and promote plant growth. Li et al. (2022) have shown that the synergistic effect of controlled-release urea slow-release nutrients and FA-promoted growth can improve the soil nitrogen cycle microbial community, reduce nutrient loss, and increase wheat yield. In addition, studies have shown that the combined application of urea and FA solution significantly increased soil nutrient availability (Gao et al. 2022). Our results showed that the application of FA significantly increased N accumulation grains (Figure 2A), which may be due to the FA reducing soil N loss and enhancing plant N uptake. The soil nutrient concentrations at maturity results showed that FA application decreased the total N concentrations (except for W2C1) but increased the available N concentrations compared with CK (Table 5). It was indicated that applying FA could increase the available N supply to the plants, as demonstrated by Liu et al. (2022) and Selim and Mosa (2012).

Moreover, it has been reported that HA extracted from lignite has a relatively high affinity for nitrate and the ability to influence its behaviour in soils, which could be utilised in the soil to participate in nitrate uptake and assimilation (Klučáková 2010). HA fertiliser application could efficiently decrease soil salinity and improve soil nutrient availability in coastal saline soil by affecting the bacterial and fungal community structure in the harvest stage (Liu et al. 2019a). Therefore, we speculate that FA may improve soil nutrient availability by changing soil microbial abundance and reducing nutrient leaching while also regulating gene expression and physiological metabolism related to nutrient uptake and transport in plants, thereby promoting crop nutrient uptake and improving nutrient use efficiency.

The concentrations of Fe and Zn in wheat grains are essential indexes of grain quality closely related to human health. Therefore, improving the con-

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centrations of Fe and Zn in wheat grain is of great significance for improving human health through nutritional fortification (Nikolic et al. 2016). Studies have shown that biostimulants or bioactive molecules can improve plant uptake of microelements. Xue et al. (2018) found that applying nanochitin can significantly increase Fe and Zn wheat grain concentrations. It has been shown that HS is involved in plant Fe nutrition *via* strategies I and II, thereby improving plant Fe nutrition. Moreover, Zanin et al. (2019) found that HS improved plant Fe nutrition through metal complexation. In addition to forming metal chelates with Fe to increase plant Fe availability, it has been observed that HA upregulates the expression of Fe transporters in the roots and leaves of rapeseed (Billard et al. 2014). Our results have also shown that applying FA provides remarkable advantages in increasing the grain Fe concentration by 22.83~106.68% compared to the control. We speculated that this advantage might be attributed to the fact that FA can increase Fe availability in the soil while promoting gene expression in plants related to Fe uptake. Of course, relevant molecular biology experiments need to verify these inferences in the future. Compared with Fe concentration, FA showed a weaker effect on increasing grain Zn concentration in this study. This difference may be related to the different abilities of FA to activate soil Zn in different soil types. Therefore, applying FA to enhance the Fe nutrition of wheat grains can allow for significant effects, and the effects of small-MW FAs are much higher than those of medium- and large-MW FAs.

In conclusion, applying different MW FAs can significantly increase the plant biomass, grain yields, nutrient uptake, and N translocation of winter wheat and that grain Fe concentrations can be significantly increased, which has advantages in improving wheat grain nutrition. The improvements of these indexes by different MW FAs varied. There was a significant interaction between the MW and application rate of FA, and small MW FAs have more obvious advantages in improving grain yield, nutrient uptake efficiency, and grain quality.

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