https://doi.org/10.17221/320/2024-PSE

Enhanced maize yield and nitrogen efficiency with low molecular weight fulvic acid: insights into chlorophyll *a/b* ratio and nitrogen metabolising enzyme activity

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Citation: Li F.G., Zhang S.J., Chai L.H., Guo Z.Q., Li P.P., Han Y.L., Wang Y. (2024): Enhanced maize yield and nitrogen efficiency with low molecular weight fulvic acid: insights into chlorophyll *a*/*b* ratio and nitrogen metabolising enzyme activity. Plant Soil Environ., 70: 632–643.

Abstract: This study investigates the impact of various molecular weights (MWs) of fulvic acid (FA) on maize growth, grain yield, and nutrient uptake under different nitrogen levels (NLs). A 2 × 3 balanced design was employed, with high (0.2 g N/kg) and low (0.05 g N/kg) NLs, and three FA MW ranges (W1 ≤ 3 000 D, 3 000 < W2 ≤ 10 000 D, W3 > 10 000 D) were applied at 25 mg/kg in soil. Significant interactions between NLs and FA MWs were observed in chlorophyll *a*/*b* ratio, nitrate reductase and glutamate dehydrogenase activities, nitrogen content, and nitrogen uptake efficiency. Overall, under different NLs, FA application reduced chlorophyll *a*/*b* ratio, increased nitrogen metabolism enzyme activities, promoted maize growth, and thereby improved grain yield and nitrogen fertiliser uptake efficiency. Additionally, the promotion effect of low MW FA on these indicators outweighed that of high MW FA, yet the latter exhibited a more pronounced effect on increasing grain nitrogen concentration. Structural equation model analysis revealed direct effects of chlorophyll content, nitrogen accumulation, nitrogen uptake efficiency, NLs and FA MWs on maize grain yield, with FA MWs negatively impacting yield.

Keywords: low nitrogen stress; nitrogen utilisation; plant growth; *Zea mays* L.

Nitrogen (N), a fundamental element in essential biological macromolecules like proteins, nucleic acids, and chlorophyll, is vital for plant growth and development. Maize cultivation places a high nitrogen demand, with the supply of nitrogen fertiliser significantly influencing maize yield (Guo et al. 2021). Insufficient nitrogen fertiliser supply can lead to yellowing of maize leaves, reduced photosynthesis, stunted plant growth, shortened ears, and reduced nitrogen concentration, all of which have a negative impact on maize yield and quality (Yu et al. 2016, Wei et al. 2016). Research indicated that the leaf growth and photosynthesis of maize were enhanced by increasing nitrogen fertiliser application, and

Supported by the National Key R&D Program of China, Grant No. 2018YFD0200600; by the Key Scientific Research Projects of Higher Education Institutions in Henan Province, Grant No. 23A210011; and by the Key Scientific and Technological Program in Henan Province, Project No. 232102111028.

https://doi.org/10.17221/320/2024-PSE

the grain yield was significantly increased (Li et al. 2023). However, the pursuit of high yields has led to widespread overapplication of nitrogen fertiliser in maize production, resulting in poor nitrogen utilisation efficiency, decreased grain quality, and nitrogen loss, ultimately causing damage to the environment (Wu et al. 2022). Therefore, it is imperative to explore environmentally friendly and sustainable methods to enhance nitrogen fertiliser efficiency, reduce application rates, and ensure high maize yields while promoting environmental protection in agriculture.

Numerous studies have demonstrated that biostimulants exhibit plant hormone-like activities, enhancing nutrient uptake and utilisation, stimulating crop growth, and increasing yield (Bulgari et al. 2015, Li et al. 2022a). Biostimulants, such as humic acids, free amino acids, seaweed extracts, and other compounds, including fulvic acid (FA), play pivotal roles in agricultural applications by serving as plant growth regulators. FA, derived from organic sources through microbial decomposition and various geochemical processes, stands out as a potent growth enhancer due to its smaller molecular weight, water solubility, and diverse functional group compositions (Calvo et al. 2014, Votolin et al. 2022). In agricultural production, FA, can promote plant growth, enhance plant resistance to biotic and abiotic stress, and improve crop yield and quality (Drobek et al. 2019, Rathor et al. 2023). It has shown that the application of FA under low nitrogen stress can promote wheat root growth and increase nitrogen uptake (Yu et al. 2023). Under drought conditions, FA can improve the tolerance of maize to oxidative stress and increase grain yield (Anjum et al. 2011). Moreover, FA is also extensively utilised in economic crop cultivation, particularly for enhancing their quality (He et al. 2022, Jiang et al. 2023). Furthermore, FA contributes to enhanced nitrogen uptake and utilisation in plants (Li et al. 2022b, De Beer et al. 2023, Liang et al. 2023).

Due to its substantial impact on crop growth and yield enhancement, FA has increasingly gained widespread usage in agriculture. However, current research on the influence of FA on nitrogen use efficiency and its effectiveness under varying nitrogen supply levels remains underexplored. In addition, as a macromolecular substance, FA exhibits a wide range of MW, leading to substantial variations in its molecular structure across different MWs. These factors will affect the effectiveness of FA as a growth regulator to improve crop nitrogen use efficiency and reduce fertiliser application (Piccolo et al. 1992, Qin et al. 2016, Nardi et al. 2021). This study employed a 2 × 3 experimental design with varying NLs and FA MWs to investigate the effects of different FA MWs on maize growth and nutrient uptake efficiency under different nitrogen conditions. By examining maize biomass, yield, nitrogen metabolism key enzyme activities, and other pertinent indicators coupled with a structural equation model analysis method, this study delves into the interplay among NLs, FA MWs, and maize indicators. These findings serve as a reference for the judicious utilisation of FA, effectively enhancing nitrogen fertiliser utilisation efficiency and alleviating environmental pressures.

MATERIAL AND METHODS

Extraction and classification of FA. 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 potassium FA, water, and resin were mixed and stirred for 5 h at the mass ratio of 1:10:20 for ion exchange, potassium ions were removed, and FA solution was obtained after filtration (Lamar et al. 2014). The FA solutions with molecular weights less than 3 000 D (W1), 3 000~10 000 D (W2) and greater than 10 000 D (W3) were achieved through dialysis using dialysis bags with molecular weight cutoffs of 3 000 D and 10 000 D, as described by Liang et al. (2023).

Experimental design. The pot experiment was carried out at Henan Agricultural University, located at 34°48'N, 113°39'E. The tested soil was loamy fluvo-aquic soil taken from the Scientific Observation Station for Nutrition and Fertilisation of Wheat and Maize Rotation in North China of the Ministry of Agriculture (Xuchang, Henan, China). The basic physical and chemical properties of the soil were as follows: pH, 7.8; total nitrogen 1.4 g N/kg; alkali-hydrolysed nitrogen 86.8 mg N/kg; available phosphorus 6.5 mg P/kg; available potassium 81.63 mg K/kg. The soil was air-dried and sieved, and then 15.0 kg of soil was weighed and placed into each polyethylene pot (30 cm diameter, 30 cm height). The N, P and K fertilisers were derived from urea (46% N), potassium dihydrogen phosphate and potassium chloride, respectively, with equal amounts of 66 mg P/kg and 170 mg K/kg used. High nitrogen (HN, 0.2 g N/kg) and low nitrogen (LN, 0.05 g N/kg) treatments were established. Maize cultivar Xianyu 335 was sown with 3 seeds per pot on June 20, 2021. Upon reaching the

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V3, one plant per pot was retained. Subsequently, FA was dissolved in 1 L of distilled water and applied to the soil at a rate of 25 mg/kg soil. Distilled water (W0) served as the control, with a total of 8 treatments established: HNW0, HNW1, HNW2, HNW3, LNW0, LNW1, LNW2, LNW3, each with 8 replicates. During the three-month growth period, the pots were placed outdoors. When it rained, the pots were covered with a rain shelter. Weeding, watering, and pest control were diligently implemented throughout the experiment.

Sampling methods. Samples were collected at the silking stage (growing period 60 days) and mature stage (growing period 90 days). At the silking stage, three plant pots were selected per treatment, and 5 cm long leaves were cut from the middle of the ear leaves (away from the main vein) and stored at –80 °C for enzyme activity and chlorophyll content analysis. The above-ground plant parts at the silking stage were divided into leaves and stalks (including ears). The samples were subjected to inaction at 105 °C for 30 min and then dried at 65 °C until a constant weight was achieved. Subsequently, they were weighed to measure the biomass. At maturity, three replicates were selected for each treatment, and the plants were divided into leaves, stalks and ears, which were dried and weighed, respectively. The nitrogen content of dried plant samples was determined after grinding.

Measurement indicators and methods. Chlorophyll content was determined using ethanol acetone mixture spectrophotometry (Li et al. 2020). Fresh leaves stored at –80 °C were ground into powder in liquid nitrogen. A 0.1000 g portion of the sample was weighed and put into a 10 mL centrifuge tube, and 5 mL of the mixed extraction agent (V acetone: V ethanol = 1:1) was added. After shaking, the sample was placed at 25 °C in the dark for 24 h and then centrifuged at 3 000~4 000 *r*/min for 10 min to obtain the extract solution. The absorbance was measured at 663 nm and 645 nm. Chlorophyll content is calculated according to the following formula:

$$\text{Chl } a = 12.7 \times \text{A663} - 2.69 \times \text{A645}; \text{ Chl } b = 22.9 \times \times \text{A645} - 4.68 \times \text{A633} \text{ (Arnon 1949).}$$

Nitrogen concentration was determined by digestion with H_2SO_4-H_2O_2, followed by analysis using a Kjeldberg nitrogen analyser (Gerhardt Analytical Systems, Königswinter, Germany).

Nitrogen uptake efficiency = nitrogen accumulation per pot/nitrogen application per pot; Nitrogen partial factor productivity = grain yield per pot/nitrogen application amount per pot (Przulj and Momcilovic 2001).

The activities of nitrate reductase (NR), glutamine synthetase (GS), glutamate dehydrogenase (GDH), and glutamate synthetase (GOGAT) were evaluated using the Thermo Scientific™ Multiskan™ and measured with an FC microplate analyser (Thermo Fisher Scientific, Helsinki, Finland).

Data processing and analysis. The experimental data were compiled in Excel 2019 (Microsoft Corp., Washington, USA) and statistically analysed using IBM SPSS Statistics 20.0 software (IBM, New York, USA). GraphPad Prism 8.0.2 (GraphPad Software, Inc., San Diego, USA) was used to create a chart, and the Pearson method was used to analyse the correlation between indicators. Two-way analysis of variance (ANOVA) was employed to assess the statistical analysis of all data. Multiple comparisons of mean values for main and interaction effects were performed using the least significant difference (*LSD*) and Duncan's method test ($P < 0.05$).

Structural equation modelling (SEM) was constructed using IBM SPSS AMOS 24 software (IBM, New York, USA) to investigate the direct and indirect effects of MW of FA and NL on nitrogen uptake efficiency as well as yield in maize. The model had seven predictors: MW, NL, chlorophyll, enzyme activity, nitrogen accumulation, nitrogen uptake efficiency, and yield. Model fit was assessed by the model's chi-square degrees of freedom ratio and its associated *P*-value, comparative fit index (CFI), standard fit index (NFI), and root mean square of residuals (RMR).

RESULTS

Effects of FA with different molecular weights on chlorophyll content of maize leaves at high and low nitrogen levels. The interaction effects of NL and MW of FA on chlorophyll *a*/*b* ratio and leaf nitrogen concentration were significant ($P < 0.05$ and $P < 0.01$, respectively); the main effect of NL on chlorophyll *a* and chlorophyll *b* content of maize at the silking stage reached a significant level ($P < 0.05$); and the main effect of MWs of FA on chlorophyll *b* content reached a significant level ($P < 0.01$) (Figure 1).

Under the same nitrogen level, the application of FA with different MWs had no significant effect on chlorophyll *a* content in maize leaves (Figure 1A) but significantly increased the content of chlorophyll *b* and decreased the ratio of chlorophyll *a*/*b* (except LNW3). The chlorophyll *b* content decreased with the increase of FA MW (Figures 1B, C). The highest

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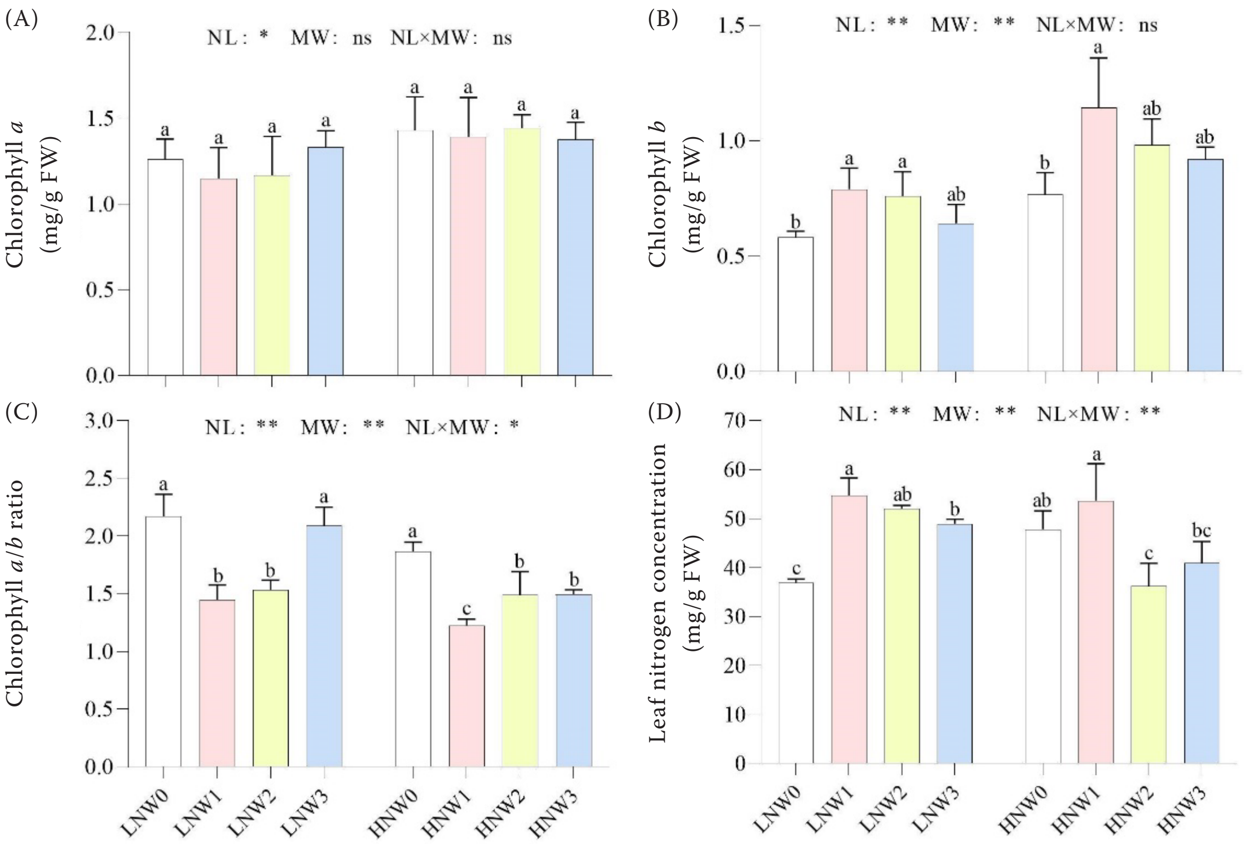

Figure 1. Chlorophyll and nitrogen content of maize leaves under different treatments. (A) Chlorophyll *a*; (B) chlorophyll *b*; (C) chlorophyll *a/b* ratio, and (D) leaf nitrogen concentration. Each column represents the mean ± standard deviation of at least three independent biological replicates. Different letters indicate the significant differences between treatments at $P < 0.05$ levels, according to the one-way ANOVA test followed by Duncan's test. 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 ($P < 0.05$). NL – nitrogen level; MW – molecular weight; NL × MW – the interaction effect of both. $^*P < 0.05$; $^{**}P < 0.01$; ns – there was no significant difference; FW – fresh weight; LN – low nitrogen (0.05 g N/kg); HN – high nitrogen (0.2 g N/kg); fulvic acid solutions: W1 – 3 000 D; W2 – 3 000 ~ 10 000 D; W3 – greater than 10 000 D

chlorophyll *b* content and the lowest chlorophyll *a*/*b* ratio were observed with W1 applied at both NLs (Figure 1D). In addition, FA application significantly increased the nitrogen concentration of maize leaves under low nitrogen.

Effects of different molecular weight FA on enzyme activity of maize leaves under high nitrogen and low nitrogen levels. The interaction effects of NL and MW of FA on the activities of nitrate reductase and glutamate dehydrogenase in maize leaves reached a significant level ($P < 0.01$). The main effects of both glutamine synthetase and glutamate synthetase activities were significant ($P < 0.01$).

The application of different MWs of FA significantly increased NR activities at different NLs, and the enzyme activity increased with the increase of FA MW, with significant differences between different MWs (Figure 2A). The application of different MWs of FA significantly increased GS, GOGAT (except for HNW3), and GDH (except for HNW3) enzyme activities in maize leaves under both low and high nitrogen treatments as compared with control (Figures 2B–D). Different from NR, the activities of these three enzymes decreased with the increase of FA MW, and the enzyme activity following W1 application reached its peak.

Effects of FA with different molecular weights on biomass, yield and nutrient uptake of maize under high and low nitrogen levels. The results of the two-way ANOVA showed that the interaction effects of NL and MW of FA on nitrogen accumulation (NA-L-S, NA-S-S, NA-L-M, NA-S-M, NA-G), grain

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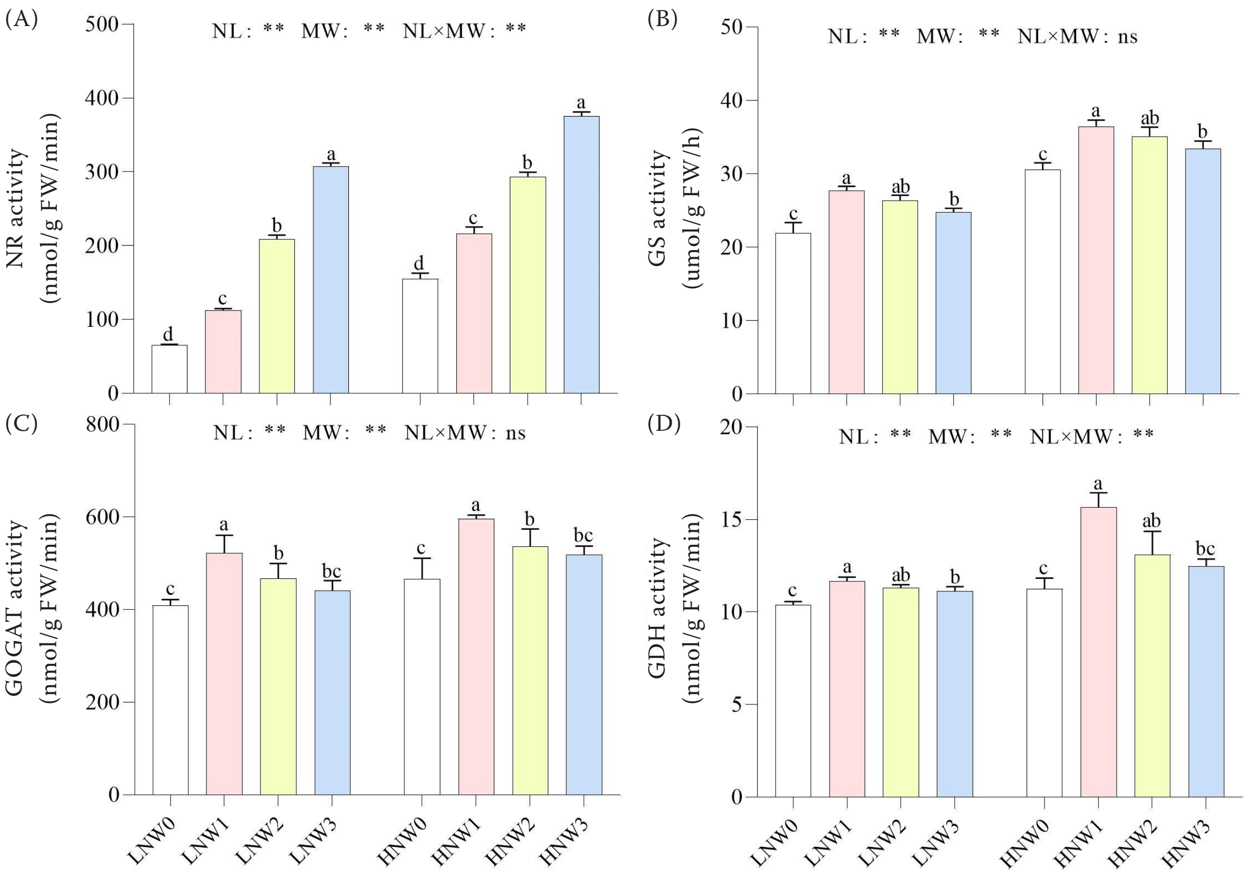

Figure 2. Enzyme activity of maize leaves under different treatments. (A) Nitrate reductase (NR) activity; (B) glutamine synthetase (GS) activity; (C) glutamate synthetase (GOGAT) activity, and (D) glutamate dehydrogenase (GDH) activity. Each column represents the mean ± standard deviation of at least three independent biological replicates. Different letters indicate the significant differences between treatments at $P < 0.05$ levels, respectively, according to the one-way ANOVA test followed by Duncan's test. 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 ($P < 0.05$). NL – nitrogen level; MW – molecular weight; NL × MW – the interaction effect of both. *$P < 0.05$; **$P < 0.01$; ns – there was no significant difference; FW – fresh weight; LN – low nitrogen (0.05 g N/kg); HN – high nitrogen (0.2 g N/kg); fulvic acid solutions: W1 – 3 000 D; W2 – 3 000 ~ 10 000 D; W3 – greater than 10 000 D

nitrogen concentration (NC-G), nitrogen uptake efficiency (N-UE) and nitrogen partial factor productivity (N-PFP) in different parts of the maize aboveground parts were significant ($P < 0.01$). The main effect of NL on aboveground biomass (B-L-S, B-L-M, B-S-M, B-AG-M) and grain yield was significant ($P < 0.01$). The main effect of MW of FA on maize aboveground biomass (B-S-S, B-AG-S, B-AG-M) and grain yield was significant ($P < 0.01$) (Table 1).

Under the two different NLs, the application of different MWs of FA increased the aboveground biomass of maize during both silking and mature stages, and the increase decreased with the increase of FA MW. However, this increase diminished as the MW of FA increased. Notably, the W1 treatment exhibited significantly higher biomass compared to other treatments. The aboveground biomass of LNW1 and HNW1 treatments increased by 11.0% and 8.5%, respectively, compared to the control at the silking stage, and increased by 22.6% and 23.5%, respectively, at the mature stage. Furthermore, under low nitrogen conditions, the application of different MWs of FA significantly increased maize grain yield by 39.3% to 63.1% compared with control. However, this increase declined as the MW of FA increased, with the effect of the W1 treatment significantly higher than other treatments. Under high nitrogen conditions, grain yield increased by 4.8% to 40.3%

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Table 1. Effects of different treatments on biomass, yield and nutrient uptake of maize

Test indicator	Low nitrogen				High nitrogen				Statistical analysis		
	W0	W1	W2	W3	W0	W1	W2	W3	NL	MW	NL × MW
B-L-S (g/plant)	23.16^{a}	24.29^{a}	22.64^{a}	23.12^{a}	27.12^{a}	29.63^{a}	29.11^{a}	26.56^{a}	**	ns	**
B-S-S (g/plant)	58.47^{b}	66.32^{a}	63.96^{ab}	60.15^{ab}	59.74^{ab}	64.58^{a}	58.98^{b}	55.56^{b}	ns	**	ns
B-AG-S (g/plant)	81.63^{b}	90.60^{a}	86.60^{ab}	83.27^{ab}	86.86^{b}	94.21^{a}	88.09^{b}	82.12^{b}	ns	*	ns
B-L-M (g/plant)	19.10^{a}	21.16^{a}	19.30^{a}	19.14^{a}	21.86^{a}	25.35^{a}	22.99^{a}	24.00^{a}	**	ns	ns
B-S-M (g/plant)	55.43^{a}	55.54^{a}	51.12^{a}	48.95^{a}	60.27^{a}	65.47^{a}	61.01^{a}	57.09^{a}	**	ns	ns
B-AG-M (g/plant)	110.76^{b}	135.78^{a}	120.99^{b}	118.59^{b}	144.89^{b}	178.89^{a}	155.63^{b}	146.87^{b}	**	**	ns
Yield (g/plant)	36.23^{b}	59.08^{a}	50.57^{a}	50.47^{a}	62.78^{b}	88.07^{a}	71.63^{b}	65.78^{b}	**	**	ns
NA-L-S (g)	0.86^{b}	1.33^{a}	1.28^{a}	1.46^{a}	1.30^{a}	1.37^{a}	1.38^{a}	1.18^{a}	ns	*	**
NA-S-S (g)	2.45^{a}	2.54^{a}	2.64^{a}	2.37^{a}	2.43^{bc}	3.82^{a}	3.26^{ab}	2.02^{c}	*	**	**
NA-L-M (g)	0.65^{c}	0.72^{b}	0.64^{c}	0.88^{a}	0.85^{b}	0.86^{b}	1.05^{a}	1.09^{a}	**	**	**
NA-S-M (g)	2.31^{c}	2.62^{b}	2.67^{b}	3.86^{a}	4.26^{a}	4.68^{a}	4.76^{a}	4.71^{a}	**	**	**
NC-G (%)	3.46^{d}	5.50^{c}	6.47^{b}	7.63^{a}	7.46^{c}	13.19^{b}	14.80^{a}	14.45^{a}	**	**	**
NA-G (g)	1.25^{b}	3.51^{a}	3.27^{a}	3.72^{a}	4.95^{c}	11.62^{a}	10.59^{ab}	9.51^{b}	**	**	**
N-UE (g/g)	0.56^{c}	0.87^{b}	0.88^{b}	1.17^{a}	0.34^{c}	0.58^{a}	0.54^{ab}	0.50^{b}	**	**	**
N-PFP (g/g)	50.80^{b}	78.78^{a}	67.44^{a}	67.30^{a}	20.93^{b}	29.36^{a}	23.88^{b}	21.93^{b}	**	**	**

B-L-S – leaf biomass at silking stage; B-S-S – stem biomass at silking stage; B-AG-S – aboveground biomass at silking stage; B-L-M – leaf biomass at maturity stage; B-S-M – stem biomass at maturity stage; B-AG-M – aboveground biomass at maturity stage; NA-L-S – nitrogen accumulation in leaves at silking stage; NA-S-S – stem nitrogen accumulation at silking stage; NA-L-M – nitrogen accumulation in leaves at maturity stage; NA-S-M – stem nitrogen accumulation at maturity stage; NC-G – grain nitrogen concentration; NA-G – grain nitrogen accumulation at maturity stage; N-UE – nitrogen uptake efficiency; N-PFP – nitrogen partial factor productivity; NL – nitrogen level; MW – molecular weight; NL × MW – interaction effect of the two; Different letters after the same column of data indicate significant differences between treatments ($P < 0.05$); $^{*}P < 0.05$; $^{**}P < 0.01$; ns – there was no significant difference; fulvic acid solutions: W1 – 3 000 D; W2 – 3 000 ~ 10 000 D; W3 – greater than 10 000 D

compared to the control; only the W1 treatment was significantly higher than the W0 treatment.

Various MWs of FA markedly enhanced the nitrogen accumulation in maize leaves at the silking stage (NA-L-S) and stem nitrogen accumulation at the mature stage (NA-S-M) under low nitrogen conditions, exhibiting increments of 48.8% to 69.8% and 13.4% to 67.1% respectively compared to the control. Application of FA with different MWs significantly increased nitrogen concentration in maize grains under both low and high nitrogen levels, with the effect of high MW FA surpassing that of low MW FA. Specifically, under low and high nitrogen conditions, compared with the control, the application of FA increased grain nitrogen content by 59.0% to 120.5% and 76.8% to 98.4%, respectively, and the accumulative nitrogen levels in grains increased significantly. Furthermore, FA application notably improved nitrogen fertiliser uptake efficiency in maize under both low and high nitrogen conditions, resulting in increases of 55.3% to 108.9% and 47.1% to 70.6%, respectively, compared with the control. Specifically, under low nitrogen conditions, the application of FA with different MWs significantly augmented nitrogen fertiliser partial factor productivity (N-PFP) by 32.5% to 55.1% compared to the control. While FA application under high nitrogen conditions increased N-PFP, only the W1 treatment exhibited a significant difference compared to the control.

Overall, the application of different MWs of FA under different NLs exhibited varying degrees of enhancement in maize plant biomass and grain yield. The influence of high MW FA on grain nitrogen concentration surpassed that of low MW FA. However, low MW FA (W1) demonstrated notably superior nitrogen uptake efficiency and nitrogen partial factor productivity compared to W2 and W3 under both low and high nitrogen conditions.

Correlation analysis and cluster analysis of different molecular weights of FA on maize indexes under high

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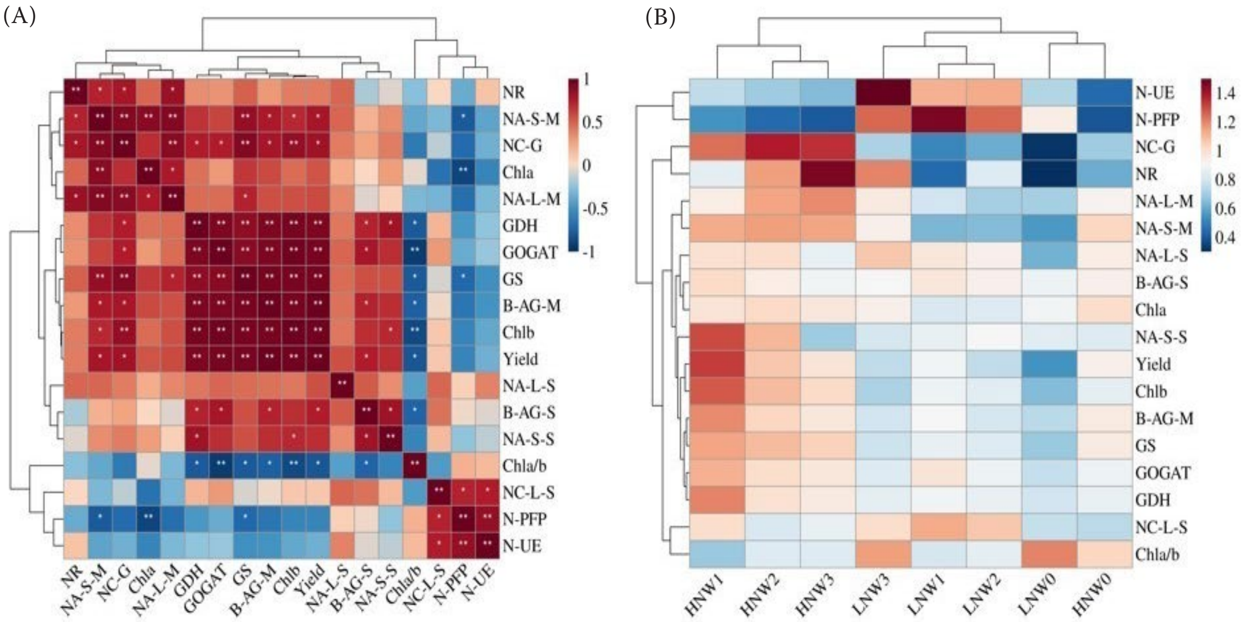

Figure 3. (A) Correlation analysis and (B) cluster analysis of indicators of maize under different treatments. B-AG-S – aboveground biomass at silking stage; B-AG-M – aboveground biomass at maturity stage; NC-L-S – leaf nitrogen concentration at silking stage; NC-G – grain nitrogen concentration; NA-L-S – nitrogen accumulation in leaves at silking stage; NA-S-S – stem nitrogen accumulation at silking stage; NA-L-M – nitrogen accumulation in leaves at maturity stage; NA-S-M – stem nitrogen accumulation at maturity stage; N-UE – nitrogen uptake efficiency; N-PFP – nitrogen partial factor productivity; Chl*a* – chlorophyll *a*; Chl*b* – chlorophyll *b*; Chla/b – chlorophyll *a/b* value; NR – nitrate reductase; GS – glutamine synthetase; GOGAT – glutamate synthetase; GDH – glutamate dehydrogenase; LN – low nitrogen (0.05 g N/kg); HN – high nitrogen (0.2 g N/kg); fulvic acid solutions: W1 – 3 000 D; W2 – 3 000 ~ 10 000 D; W3 – greater than 10 000 D

and low nitrogen levels. The correlation analysis results (Figure 3A) showed that nitrogen concentration, nitrogen accumulation and aboveground biomass of maize plants were significantly positively correlated with chlorophyll and the activities of the four enzymes. Moreover, maize yield was positively correlated with aboveground biomass, chlorophyll content, enzyme activities of GS, GOGAT and GDH, as well as nitrogen accumulation.

The cluster analysis results (Figure 3B) showed that GS, GOGAT, and GDH formed an initial cluster, followed by their grouping with chlorophyll content, yield, and other indicators. NR, however, clusters separately with grain nitrogen concentration. Notably, there were substantial differences between FA-treated and untreated (W0) groups, leading to their classification into distinct clusters. Within the high nitrogen treatment groups, HNW2 and HNW3 are preferentially clustered, subsequently followed by their integration with HNW1. Conversely, the low nitrogen treatment groups (LNW1 and LNW2) exhibited a similar preferential clustering, followed by their integration with LNW3.

Structural equation model of nitrogen level, molecular weight of FA and maize indexes. By constructing a structural equation model (SEM), this study investigated the direct and indirect effects of MW of FA and NL on maize nitrogen uptake efficiency and yield. The SEM analysis (Figure 4) revealed that enzyme activity ($\beta = 0.38$, $P < 0.001$), chlorophyll ($\beta = 0.16$, $P < 0.01$) and nitrogen accumulation ($\beta = 0.71$, $P < 0.001$) significantly affected nitrogen accumulation and yield. Moreover, the direct effect of NL on yield was significantly positively correlated ($\beta = 0.91$, $P < 0.01$), while its direct effect on nitrogen uptake efficiency showed a significant negative correlation ($\beta = -1.42$, $P < 0.001$). However, NL positively impacted nitrogen uptake efficiency by affecting enzyme activity ($\beta = 0.51$, $P < 0.001$). Additionally, the MW of FA positively influenced nitrogen uptake efficiency and yield by influencing enzyme activity ($\beta = 0.86$, $P < 0.001$), chlorophyll content ($\beta = 0.10$, $P < 0.01$) and nitrogen accumulation ($\beta = 0.35$, $P < 0.001$). The application of FA improved nitrogen uptake efficiency and yield. Notably, the direct effect of MW

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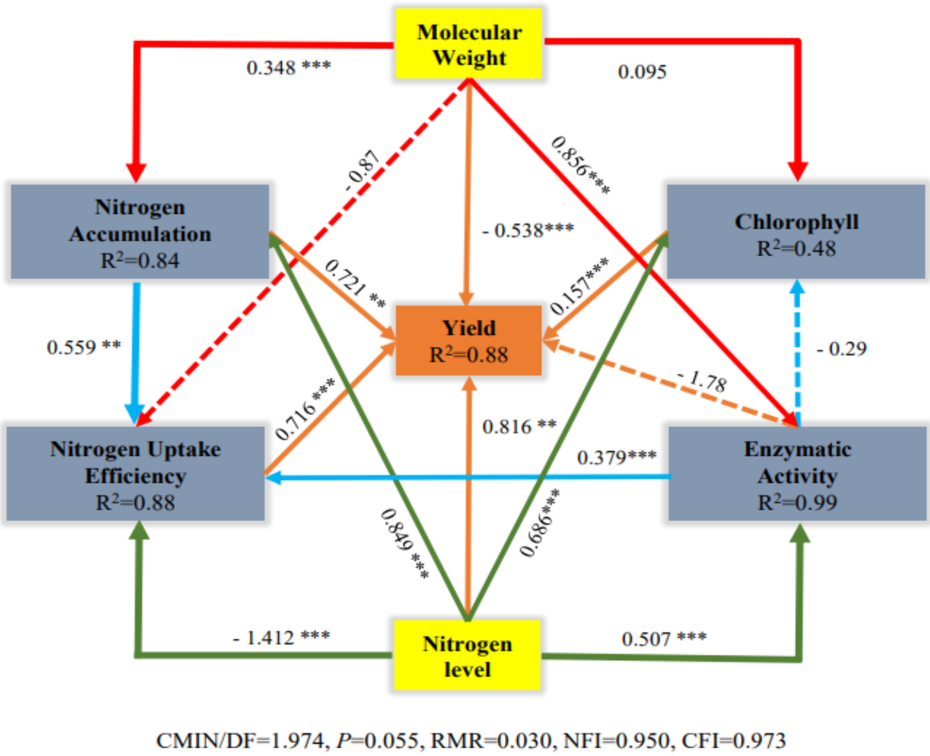

Figure 4. Structural equation model of nitrogen level, molecular weight of fulvic acid and each index of maize. The solid line indicates a significant association ($P < 0.05$), while the dashed line indicates a lack of statistical significance ($P > 0.05$). The values next to the solid and dashed arrows represent normalised path coefficients (β), with positive values representing positive and negative values representing negative correlations. The boxes represent the variables included in the model, while the numbers represent the proportion of variation (R^2) explained by the model; RMR – root mean square of residuals; NFI – standard fit index; CFI – comparative fit index

of FA on yield was significantly negatively correlated (β = –0.53, $P < 0.001$), indicating that the increase of MW of FA may lead to a decrease in yield.

DISCUSSION

FA, characterised by its water-soluble, low molecular weight fraction of humic acid with abundant active functional groups, possesses various special physicochemical properties and serves diverse physiological roles in plants. Therefore, it has gained widespread recognition as an effective biostimulant and fertiliser enhancer in agriculture (Gong et al. 2022). Extensive research has demonstrated that FA exerts positive effects on plant growth, development, stress resistance, and yield enhancement. For instance, FA has been shown to reduce transpiration rate, thereby improving water use efficiency and enhancing crop drought resistance through the regulation of stomatal opening (Yang et al. 2017). Furthermore, by improving the availability of soil nutrients, mitigating soil salinity and increasing the chlorophyll and proline content in crops, FA contributes to improved salt tolerance in plants (Zhang et al. 2023). Additionally, the presence of hydroxyl, carboxyl, benzene-carboxylic acid and other functional groups in FA promotes disease resistance in crops (Yigit and Dikilitas 2008). Research has indicated significant variations in plant growth promotion among different MWs of FA (Juncan et al. 2021, Liang et al. 2023). Nardi et al. (2007) observed that the lower the MW of humic acid applied, the higher the glycolysis and tricarboxylic acid cycle-related enzyme activities in maize seedlings. Similarly, the present study showed that different MWs of FA can promote the growth and yield of maize under different NLs, with noticeable MW-dependent effects. Specifically, the promotion effect of low MW FA (W1) was significantly superior to that of other MWs, which was consistent with the previous findings in wheat (Liang et al. 2023).

Chlorophyll plays a central role in plant photosynthesis by capturing light energy. Chlorophyll *a*

https://doi.org/10.17221/320/2024-PSE

is the primary pigment responsible for light energy capture and electron transport in photosynthesis, while chlorophyll *b* complements this by transferring energy to chlorophyll *a*, expanding the range of light absorption (Yamasato et al. 2005, Zhang et al. 2017). The optimal ratio of chlorophyll *a* to chlorophyll *b* is important for enhancing plant photosynthetic efficiency (Neidhardt et al. 1998), which can be influenced by various factors, including plant species, light intensity, temperature, and nutrient status (Johnson et al. 1993, Kitajima and Hogan 2003, Esteban et al. 2015, Yamori 2016). For example, the chlorophyll *a*/*b* ratio in citrus leaves is negatively correlated with the leaf nitrogen content (Bondada and Syvertsen 2003). In maize, supplying adequate nitrogen fertiliser under salt stress conditions can increase chlorophyll content in leaves, thereby affecting the chlorophyll *a*/*b* ratio and promoting growth (Akram 2014). Research on higher-yielding hybrid maize cultivars highlights the importance of elevated chlorophyll content, a lower chlorophyll *a*/*b* ratio, increased photosynthetic capacity, and prolonged photosynthetic duration (Yan et al. 2021).

The findings of this study revealed that under LN stress, there was a significant decrease in the chlorophyll *b* content of maize leaves at the silking stage, accompanied by an increase in the chlorophyll *a*/*b* ratio. However, the application of different MWs of FA significantly elevated the chlorophyll *b* content of maize leaves under LN conditions, while concurrently reducing the chlorophyll *a*/*b* ratio compared to the control (Figures 1B–C). This application mitigated the adverse effects of low nitrogen stress on maize leaf chlorophyll content and improved leaf photosynthesis. Additionally, under HN conditions, FA application also notably increased chlorophyll *b* content and decreased the chlorophyll *a*/*b* ratio (Figure 1B–C), thus promoting the improvement of photosynthesis. Consequently, FA application under different nitrogen levels demonstrated an ability to enhance leaf photosynthesis by increasing chlorophyll *b* content and decreasing chlorophyll *a*/*b* ratio, thereby promoting plant growth and yield.

Nitrogen metabolism is essential for plant growth, development, and yield, relying on enzymes such as GS, GOGAT, GDH and NR (McAllister et al. 2012). Their activity significantly impacts plant nitrogen use efficiency (Nasar et al. 2022). Various factors influence enzyme activity, including nitrogen supply level, light, temperature, pH, and nitrogen source. Under stress, such as low nitrogen, the activities of nitrogen assimilation-related enzymes are inhibited, impacting nitrogen metabolism and plant growth. For instance, low nitrogen stress reduces GOGAT activity in *Sophora japonica*, inhibiting ammonium assimilation (Tian et al. 2021). Drought stress diminishes NR, GS and GOGAT activities, raising ammonium concentration and causing toxicity (Pawar et al. 2015). High Na^+ and Cl^- concentrations lower NR and GDH activities, hindering nitrogen assimilation (Flores et al. 2000, Kumar et al. 2000). Growth regulators or biostimulants mitigate stress-induced enzyme inhibition, enhancing the nitrogen assimilation and promoting plant growth (Baltazar et al. 2021). This study revealed that both nitrogen level and MW of FA significantly influenced the activities of key enzymes, NR, GS, GOGAT and GDH, in nitrogen metabolism. The application of FA in both HN and LN conditions improved these enzyme activities. Additionally, different MWs of FA exhibited distinct effects on enzyme activities. Higher MW of FA exerted stronger activation on NR, whereas lower MW had the most significant activation effects on GS, GOGAT and GDH enzyme activities (Figure 2).

Most studies indicated that biomacromolecules with lower MW tend to exhibit greater biological activity. Low MW chitosan (30 kD) demonstrated heightened preservation and antifungal efficacy in cucumber (Yuan et al. 2023). Similarly, low MW polysaccharide (5.2 kD) showed superior antioxidant properties compared to high MW (15.4 kD) (Liu et al. 2010). Humic acid, as a macromolecular substance, processes a diverse structure and a broad molecular weight range. Different molecular weight fractions of humic acid contain distinct functional groups, leading to significant variations in biological activities (Antonelli et al. 2001). Despite FA being a smaller molecular component of humic acid, its molecular weight spans from several hundred to tens of thousands of daltons. Previous studies have revealed notable disparities in elemental composition, functional groups, and hydrophilicity among the three molecular weights of FA utilised in this research (Liang et al. 2023). The combined effect of these factors may contribute to the pronounced effect of low MW of FA on the key enzyme activities involved in nitrogen metabolism in maize leaves.

The accumulation of crop biomass is related to both the nutrient uptake efficiency and accumulation, serving as the foundation for biomass accumulation and yield formation (Suriyagoda et al. 2020, Muniz et al. 2021). Research indicates that the utilisation of

https://doi.org/10.17221/320/2024-PSE

FA can notably enhance nutrient uptake, especially nitrogen, thereby increasing crop yield by improving fertiliser efficiency (Verlinden et al. 2009). Studies on wheat demonstrate that combining nitrogen fertiliser with FA enhances nitrogen and phosphorus absorption, reduces nutrient loss, and elevates nitrogen use efficiency (Çimrin et al. 2010, Liu et al. 2022). Furthermore, the application of different molecular weights of FA in various soil textures significantly enhanced nitrogen uptake in wheat, thereby improving nitrogen efficiency and productivity (Liang et al. 2023). Importantly, FA application enhances nitrogen uptake efficiency, accumulation, and productivity in maize across varied nitrogen levels, leading to a substantial increase in grain nitrogen concentration (Table 1). The two-way ANOVA results revealed a significant interaction effect between the MW of FA and NL on the nitrogen uptake efficiency of wheat. The SEM analysis indicated that the MW of FA and NL had significant direct and indirect effects on the yield. The application of FA could increase the yield of maize with different NLs, yet as the increase of FA MW, the yield decreased.

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Received: June 12, 2024
Accepted: August 5, 2024
Published online: September 7, 2024