Reduced Application of Nitrogen Fertilizer Affects Nitrogen Metabolism of Leaves and Maintains the Production During the Harvesting Stage in *Coreopsis tinctoria*

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Abstract

Coreopsis tinctoria, as a medicinal plant, has become popular in China in the past decade. However, basic data on NO_3^- supply related to its production and nitrogen (N) metabolism are lacking. In this study, all plants were grown to four leaves in substrate (vermiculite:perlite = 2:1), which were subsequently transferred to different reducing NO_3^- solutions. Appropriate N fertilizer application reductions (1/8N-1/2N) maintained the flower number and biomass of *Coreopsis tinctoria* during 2020–2021. N fertilizer reduction maintained the N assimilation levels of *C. tinctoria* and increased N use efficiency (NUE), one of the important reasons was that the reduction of N fertilizer did not damage the chlorophyll fluorescence system and photosynthetic system. 1/2N-1/8N treatment also maintained the activities nitrate reductase (NR), nitrite reductase (NIR), glutamine synthase (GS) and glutamate synthase (GOGAT) and the content of total amino acid and soluble protein in leaves. N reduction treatment also increased the levels of tyrosine (Thr), serine (Ser), leucine (Leu), isoleucine (Ile), lysine (Lys) and arginine (Arg) of leaves in *C. tinctoria*, and further enhances N assimilation ability. Leaf N content was significantly correlated with the activities of NR, NIR, GS, GOGAT and GDH, indicating that N metabolism was effectively regulated by N levels. In conclusion, the 1/8 N level prior to harvest could be one of the strategies for *C. tinctoria* cultivation because it minimizes planting costs and maintains nitrogen assimilation capacity and productivity.

Introduction

Coreopsis tinctoria is mainly distributed in the midwestern United States, Eastern Asia and Western Europe. It is rich in biologically active substances and is used as a raw material for tea and health products[1]. In China, Xinjiang is the main *C. tinctoria* production area, and high-quality products made from this plant are supplied to all parts of the country. In recent years, the market demand and cultivation area of *C. tinctoria* have continued to expand. However, *C. tinctoria* production in different places varies greatly, which makes the evaluation of product quality and production work confusing[2]. At present, the spatial and temporal competition of *C. tinctoria* with other chrysanthemum products and the high cost, as well as unreasonable management of fertilizer applications, seem to be one of the major challenges for profitable *C. tinctoria* production.

Nitrogen (N) is an important plant nutrient element and a fertilizer component required by plants. N is very important in regulating plant metabolism and growth[3]. This element is involved in a variety of plant physiological functions, such as enzyme activity, osmotic balance, regulation of stomatal movement, changes in photosynthesis and assimilate transport[4]. Irrational use of N is currently a controversial issue in the agricultural development of many countries worldwide. Plants absorb N from the environment, and the N balance decreases with time[5]. There are many ways to lose N fertilizer, and it is very easily leached and volatilized. The environmental pressure caused by increasing the number of N applications is becoming increasingly severe. In addition, excessive application of N fertilizer can also cause problems such as a decline in crop quality and an increase in production costs[6]. Therefore, reducing N application and increasing N use efficiency (NUE) have gradually become general tactics supported by researchers. In China, the NUE is only approximately 30%, and the percentage continues to decline[7, 8]. Thus, it is particularly important to appropriately reduce the amount of N fertilizer necessary for plant growth and yield by regulating the biological processes of plant carbon metabolism, C/N balance and N metabolism[9].

Balanced N nutrition allows plants to absorb other essential nutrients from the soil and transport them to other tissues to ultimately act on the metabolic network. For example, nitrates in the xylem of wheat move together with potassium ions and diffuse into specific tissues[10, 11], Earlier studies have found that the accumulation of amino acids and proteins in *C. tinctoria* leaves depends heavily on the N concentration. When plants are under low N stress, protein synthesis and amino acid transport systems are hindered[12]. At this time, the main enzymes involved in N assimilation, nitrate reductase (NR) and glutamine synthetase (GS), are involved in regulating the concentration of N and protein in leaves. Nitrite reductase (NIR), GS, and glutamate synthetase (GOGAT) work in tandem with NR to improve the efficiency of plant N metabolism. GS activity guarantees the process of organic N assimilation resulting from light assimilation in the chloroplast[13]. In research on soybeans, the activities of GS and GOGAT were found to be regulated by nitrate[14]. The content of amino acids in plants can be adjusted by the following methods: protein decomposition, nitrate assimilation, amino acid transport, and the use of carbon skeletons by the tricarboxylic acid cycle[15,16,17]. Therefore, this process of change may affect the development of plant reproductive structures.

Recent studies have reported that *C. tinctoria* yields can be maintained under suitable low N conditions. Without affecting the yield and economic benefits, reducing the amount of N fertilizer by more than 30% can still meet the growth needs of *C. tinctoria*[2]. Reducing the application of N fertilizer causes the plant to enter the flowering period earlier and improves the accumulation of active ingredients, which may promote competitiveness of the resulting products[2,18]. In addition, due to the long-term lack of cultivation and management experience for this crop, the high-cost fertilizer application of *C. tinctoria* is one of the main challenges limiting farmers' ability to achieve high returns, which to some extent dampens interest in *C. tinctoria* planting. Therefore, reducing the application cost of N fertilizer and shifting the harvest period may be a practical strategy for regulating the *C. tinctoria* industry. However, what happens to the N assimilation process of *C. tinctoria* after N fertilizer levels are reduced? We hypothesize that reducing N application is a feasible method to maintain the effective N metabolism and yield of *C. tinctoria*.

Results

Number of flower buds (or flowers) and biomass. The number of flowers in 2020–2021 was affected by N treatment (Fig. 2A and B). The flower bud (or flower) harvest in the S1-S3 continued to increase and then fell sharply in the S4. In addition, the performance of the N2-N4 treatments and the CK were essentially the same, and all were significantly higher than that of the N1 treatment ($P < 0.05$). The ranges of changes were 56.16–73.50 and 66.32–89.23 for the total number of flowers in the two growth stages, respectively. Notably, the total number of flowers in 2020 was greater than that in 2021.

The single-leaf biomass did not change significantly in N2-N4 compared with the CK and was significantly higher than that of the N1 treatment during all harvest stages of the two growing seasons (Fig. 2C, $P < 0.05$). The ranges of variation were 0.46–0.71 $g \cdot leaf^{-1}$ and 0.44–0.68 $g \cdot leaf^{-1}$ for the number of

flowers in the two growth stages, respectively. Similarly, the total weight of functional leaves exhibited the same pattern as the biomass of a single leaf. The range of variation in functional leaves under all treatments was 17.71–69.58 g·plant^{-1} in 2020, while it was 17.54–57.75 g·plant^{-1} in 2021. In addition, the accumulation of functional leaves in 2020 was higher than that in 2021 (Fig. 2D).

Leaf N contents. There was a positive correlation between leaf N content and N concentration in four stages of the two growing seasons (Fig. 3A). In 2020, compared with that of the CK, the leaf N content of the four N treatments (N1, N2, N3, and N4) decreased by 24.40-62.71% during the four the harvest stages. In 2021, the leaf N content of the four N treatments (N1, N2, N3, and N4) decreased by 16.8-59.22% compared with that of the CK treatment.

Chloroplast pigment, chlorophyll fluorescence, and gas exchange parameters. N treatment affected the chloroplast pigment content of *C. tinctoria* leaves ($P < 0.05$). For the harvest period, the chlorophyll a, chlorophyll b and carotenoid contents peaked at S3 and then decreased in 2020–2021 (Fig. 3B, C and D).

The chlorophyll a content of N2, N3, N4, and the CK were not significantly different in S1, S2, and S4 during 2020–2021 ($P > 0.05$). However, the chlorophyll a content of the N3, N4 and CK treatments were significantly higher than those of the N2 and N1 treatments during S3 in both harvest seasons ($P < 0.05$). The chlorophyll b and carotenoids were unaffected by the N treatment in S1-S2, while the contents under the N2-N4 treatments and the CK in S3-S4 were higher than those under the N1 treatment in 2020–2021 ($P < 0.05$). The ranges of changes were 1.64–2.48 mg·g^{-1} and 1.52–2.42 mg·g^{-1} for chlorophyll a, 0.61–0.84 mg·g^{-1} and 0.58–0.82 mg·g^{-1} for chlorophyll b, and 0.32–0.43 mg·g^{-1} and 0.33–0.47 mg·g^{-1} for carotenoids in the two growth stages, respectively.

Changes in chlorophyll fluorescence parameters were monitored experimentally to determine the effect of low N conditions on these parameters (Fig. 4). The N starvation state (N1) significantly reduced the Fv/Fm, Fv'/Fm' and qP in leaves, while slight N deficiency conditions (N2-N4) did not affect these parameters under S1-S4 in 2020–2021. In contrast, significant increases in qN were achieved under N-starvation conditions (N1 treatment), with a trend of increasing under low N concentrations and decreasing under high N concentrations in four stages in 2020–2021. Furthermore, qN did not differ among N2-N4 and the CK at S1, while the N2 treatment caused significantly lower values than the N4 (or CK) treatment ($P < 0.05$) did at S2-S4 in the two harvest seasons.

The gas exchange parameters were similar to the chlorophyll fluorescence parameters (Fig. 5). Pn tended to increase during the S1-S3, followed by a substantial decrease at S4 in the two growth seasons. Compared with those under the CK treatment, the Pn levels decreased the under N1 treatment significantly decreased ($P < 0.05$), while those under the N2-N4 treatments remained consistent with those under the CK at S1 in 2020–2021. The performance patterns of Ci, Gs and Tr in the four harvest stages in the two growing seasons were similar to that of Pn. Overall, the performance of Ci at S1-S4 in 2020–2021 showed a trend of promotion by low N and inhibition by high N. Gs and Tr under the N2-N4 treatments and the CK were not different and were higher than the values measured under the N1 treatment at S1-S4 during 2020–2021.

Soluble proteins and free amino acids. The soluble protein content increased continuously as the N concentration increased (Fig. 6A) in the four harvest stages during 2020 and 2021. Compared with that under the CK, the content under N1 decreased 14.70% and 29.26% at S1, 39.58% and 43.10% at S2, 28.98% and 18.60% at S3, and 35.24% and 32.66% at S4 during 2020–2021, respectively. N reduction treatment significantly affected the content of total free amino acids in *C. tinctoria* leaves (Fig. 6B). For the two growing seasons, the content of total free amino acids under N2-N4 and the CK were not different but were significantly higher than those under N starvation (N1) except for S2 and S4 in 2020.

In addition, individual amino acid contents in the leaves in 2020–2021 were analyzed (Table 1). The contents and accumulation patterns of asparagine (Asn), glutamate (Glu), alanine (Ala), valine (Val), tyrosine (Tyr) and phenylalanine (Phe) were similar to those of the total amino acids. In 2020, compared with those of the CK treatment, the Asn, Glu, Ala, Val, Tyr and Phe contents of the N1 treatment decreased by 12.7–42.0% at S1, 11.6–27.8% at S2, 20.5–27.4% at S3, and 8.4–46.5% at S4, while the Asn, Glu, Ala, Val, Tyr, and Phe contents of the N1 treatment decreased by 11.4–21.1% at S1, 11.1–39.3% at S2, 4.7–19.2% at S3, and 13.4–22.7% at S4 in 2021. However, the accumulation patterns of threonine (Thr), serine (Ser), glycine (Gly), leucine (Leu), isoleucine (Ile), and lysine (Lys) decreased with increasing N concentration. In 2020, compared with those of the CK treatment, the Thr, Ser, Gly, Leu, Ile, and Lys contents of the N1 treatment increased by 20.4–48.8% at S1, 19.7–26.7% at S2, 9.0-28.6% at S3, and 8.9–27.7% at S4, the contents of Thr, Ser, Gly, Leu, Ile and Lys of the N1 treatment increased by 11.4–32.7% at S1, 10.7–38.5% at S2, 12.3–29.7% at S3, and 21.8–27.5% at S4 in 2021. Notably, the proline (Pro) content was the same as that in the CK at the level of slight N deficiency (N2-N4) and was higher than that under N-starvation conditions (N1) from S1 to S4 during 2020–2021. Arginine (Arg) levels were greatest in the slightly N-deficient treatments (N3 or N4 treatments), with significantly higher levels than those under N starvation (N1) or normal-N-supply levels (CK) ($P < 0.05$). In addition, little (or no) cysteine (Cys), methionine (Met), γ-aminobutyric acid (GABA), histidine (His), and ornithine (Orn) was detected, and the levels of these amino acids were not affected by N levels at S1-S4 in 2020–2021 (Table 2).

Table 1

Effect of N treatment on the free amino acid content (mg·g^{-1}) of *C. tinctoria* leaves (a).

Year	Harvest stage	Treatment	Asn	Thr	Ser	Glu	Gly	Ala	Val	Leu	Ile	Tyr	Phe	Lys
2020														
	S1	N1	0.213b	0.165a	0.134b	0.214b	0.125a	0.201b	0.158b	0.259a	0.141a	0.167c	0.226d	0.123a
		N2	0.257a	0.159a	0.178a	0.273a	0.134a	0.234ab	0.167b	0.231b	0.145a	0.221b	0.274c	0.118a
		N3	0.261a	0.143b	0.173a	0.303a	0.114b	0.244a	0.198a	0.232b	0.121b	0.225b	0.317b	0.119a
		N4	0.272a	0.138b	0.169a	0.261a	0.117b	0.256a	0.189a	0.226b	0.122b	0.262a	0.314b	0.096b
		CK	0.275a	0.137b	0.166a	0.284a	0.084c	0.262a	0.181a	0.213c	0.114b	0.288a	0.332a	0.089b
	S2	N1	0.223b	0.143a	0.183c	0.282c	0.118a	0.226b	0.167b	0.243a	0.112a	0.167d	0.231d	0.121a
		N2	0.293a	0.142a	0.201b	0.298b	0.123a	0.284a	0.184a	0.221b	0.096b	0.221c	0.271c	0.123a
		N3	0.301a	0.125b	0.216a	0.343a	0.117a	0.293a	0.195a	0.207b	0.084c	0.225c	0.303b	0.103a
		N4	0.285a	0.116b	0.217a	0.331a	0.101b	0.286a	0.182a	0.202b	0.089c	0.262b	0.311b	0.103b
		CK	0.293a	0.113b	0.212a	0.319a	0.093b	0.293a	0.183a	0.203b	0.086c	0.288a	0.320a	0.097b
	S3	N1	0.257c	0.194a	0.243b	0.326b	0.114b	0.279c	0.127b	0.271a	0.114a	0.194b	0.365c	0.147a
		N2	0.302b	0.173b	0.251b	0.321b	0.137a	0.316b	0.156a	0.251b	0.112a	0.273a	0.374c	0.113b
		N3	0.296b	0.169b	0.294a	0.381a	0.128a	0.342a	0.163a	0.248b	0.102b	0.281a	0.412b	0.110b
		N4	0.325a	0.174b	0.288a	0.348b	0.113b	0.354a	0.153a	0.233c	0.982b	0.267a	0.417b	0.102c
		CK	0.354a	0.178b	0.291a	0.295c	0.103b	0.363a	0.157a	0.234c	0.996b	0.258a	0.452a	0.105c
	S4	N1	0.201b	0.124a	0.193b	0.223c	0.122b	0.309b	0.196b	0.249a	0.135a	0.183c	0.376c	0.141a
		N2	0.245a	0.109b	0.213a	0.243b	0.148a	0.324a	0.191b	0.219b	0.121b	0.284b	0.389c	0.135a
		N3	0.254a	0.105b	0.217a	0.268a	0.134a	0.342a	0.224a	0.208b	0.119b	0.296b	0.415b	0.127a
		N4	0.234a	0.104b	0.223a	0.283a	0.106c	0.332a	0.222a	0.201b	0.115b	0.325a	0.412b	0.107b
		CK	0.247a	0.108b	0.222a	0.269a	0.099c	0.336a	0.235a	0.195b	0.104c	0.342a	0.473a	0.102b
2021														
	S1	N1	0.256b	0.183a	0.124c	0.235b	0.136b	0.194c	0.179b	0.261a	0.112a	0.237b	0.285c	0.134a
		N2	0.261b	0.157b	0.136b	0.274a	0.146a	0.213b	0.184b	0.234b	0.110a	0.258a	0.279c	0.131a
		N3	0.281a	0.156b	0.135b	0.285a	0.128b	0.216b	0.203a	0.242b	0.105b	0.251a	0.324b	0.119a
		N4	0.282a	0.146b	0.149a	0.282a	0.127b	0.236a	0.201a	0.237b	0.102b	0.262a	0.345a	0.106b
		CK	0.289a	0.133c	0.146a	0.287a	0.113c	0.228a	0.195a	0.236b	0.096b	0.281a	0.361a	0.101b
	S2	N1	0.245b	0.169a	0.201b	0.263b	0.114b	0.223c	0.177b	0.234a	0.094a	0.188c	0.277c	0.156a
		N2	0.264a	0.161a	0.206b	0.291a	0.123a	0.277b	0.189a	0.207b	0.084b	0.236b	0.305b	0.147a
		N3	0.261a	0.141b	0.223a	0.314a	0.122a	0.294a	0.198a	0.212b	0.083b	0.242b	0.316b	0.135a
		N4	0.284a	0.122c	0.217a	0.302a	0.102b	0.311a	0.184a	0.215b	0.081b	0.257a	0.315b	0.112b
		CK	0.295a	0.122c	0.227a	0.296a	0.103b	0.303a	0.193a	0.219b	0.074c	0.263a	0.342a	0.106b
	S3	N1	0.294b	0.221a	0.264b	0.334b	0.122b	0.265b	0.127b	0.274a	0.112a	0.194b	0.365c	0.153a
		N2	0.323a	0.193b	0.265b	0.372a	0.149a	0.295a	0.136b	0.253b	0.109a	0.257a	0.374c	0.142a
		N3	0.314a	0.187b	0.291a	0.378a	0.143a	0.332a	0.163a	0.254b	0.102b	0.267a	0.412b	0.133b
		N4	0.311a	0.191b	0.283a	0.356a	0.120b	0.341a	0.153a	0.257b	0.101b	0.273a	0.417b	0.121c
		CK	0.323a	0.183b	0.286a	0.361a	0.107c	0.336a	0.147a	0.234c	0.101b	0.281a	0.452a	0.118c
	S4	N1	0.202b	0.134a	0.197b	0.221b	0.123b	0.268b	0.186b	0.245a	0.145a	0.185b	0.289c	0.167a
		N2	0.234a	0.136a	0.194b	0.259a	0.126b	0.331a	0.183b	0.226b	0.134b	0.243a	0.301c	0.158a
		N3	0.231a	0.112b	0.206a	0.269a	0.145a	0.346a	0.223a	0.209c	0.114c	0.245a	0.324b	0.154a

[a] Means followed by a same letter within the column are not significantly different at ($P < 0.05$) probability level according to Tukey's (HSD) test.

Year	Harvest stage	Treatment	Asn	Thr	Ser	Glu	Gly	Ala	Val	Leu	Ile	Tyr	Phe	Lys
		N4	0.238a	0.114b	0.204a	0.273a	0.102c	0.344a	0.213a	0.207c	0.116c	0.256a	0.321b	0.138b
		CK	0.232a	0.109b	0.217a	0.274a	0.101c	0.342a	0.216a	0.201c	0.108d	0.259a	0.374a	0.131b

[a] Means followed by a same letter within the column are not significantly different at ($P < 0.05$) probability level according to Tukey's (HSD) test.

Table 2

Effect of low nitrogen treatment on the free amino acid content (mg·g^{-1}) of *C. tinctoria* leaves (b).

Year	Harvest stage	Treatment	Cys	Met	GABA	His	Orn
2020							
	S1	N1	/	0.01a	/	0.094a	0.038a
		N2	/	0.02a	/	0.087a	0.042a
		N3	/	0.01a	/	0.093a	0.042a
		N4	/	0.02a	/	0.085a	0.036a
		CK	/	0.01a	/	0.102a	0.041a
	S2	N1	0.01a	0.04a	0.02a	0.103a	0.044a
		N2	0.01a	0.04a	0.02a	0.112a	0.046a
		N3	0.01a	0.03a	0.02a	0.109a	0.048a
		N4	/	0.04a	0.01a	0.113a	0.042a
		CK	/	0.03a	0.02a	0.113a	0.048a
	S3	N1	/	/	/	0.103a	0.04a
		N2	/	/	/	0.112a	0.04a
		N3	/	/	/	0.109a	0.05a
		N4	/	/	/	0.113a	0.04a
		CK	/	/	/	0.113a	0.04a
	S4	N1	/	0.04a	/	0.116a	0.045a
		N2	/	0.04a	/	0.121a	0.051a
		N3	/	0.03a	/	0.125a	0.046a
		N4	/	0.04a	/	0.117a	0.046a
		CK	/	0.03a	/	0.126a	0.048a
2021							
	S1	N1	/	0.01a	/	0.996a	0.042a
		N2	/	0.02a	/	1.01a	0.045a
		N3	/	0.01a	/	0.998a	0.046a
		N4	/	0.02a	/	0.994a	0.041a
		CK	/	0.01a	/	0.984a	0.042a
	S2	N1	/	0.02a	0.01	0.102a	0.043a
		N2	/	0.03a	0.02	0.105a	0.046a
		N3	/	0.03a	0.01	0.106a	0.041a
		N4	/	0.02a	0.01	0.114a	0.046a
		CK	/	0.03a	0.01	0.115a	0.048a
	S3	N1	/	/	/	0.113a	0.03a
		N2	/	/	/	0.104a	0.03a
		N3	/	/	/	0.111a	0.02a
		N4	/	/	/	0.106a	0.03a
		CK	/	/	/	0.109a	0.02a
	S4	N1	/	0.02	/	0.128a	0.045a
		N2	/	0.03	/	0.129a	0.047a
		N3	/	0.02	/	0.133a	0.047a
		N4	/	0.03	/	0.134a	0.041a

Year	Harvest stage	Treatment	Cys	Met	GABA	His	Orn
		CK	/	0.03	/	0.133a	0.043a

Nitrogen metabolism-related enzymatic activity. N levels significantly affected N metabolism in *C. tinctoria* leaves from S1-S3 in 2020 and 2021 (Fig. 7, $P < 0.05$). The activities of NR, NIR, GS, and GOGAT tended to increase with increasing N concentrations. The activities of NR, NIR, GS, and GOGAT from S1 to S4 were 137.66-370.56 μmol·min^{-1}·g^{-1} and 124.12–365.70 μmol·min^{-1}·g^{-1}, 160.23-275.45 μmol·min^{-1}·g^{-1} and 138.95-267.65 μmol·min^{-1}·g^{-1}, 27.87–51.21 U·g^{-1} and 28.45–47.86 U·g^{-1} and 751.16-1085.49 nmol·min^{-1}·g^{-1} and 722.34-944.78 μmol·min^{-1}·g^{-1} in 2020–2021, respectively. For S1-S2, the activities of NR and GS under the N2-N4 treatments and the CK were similar during 2020–2021, while the enzyme activity of NIR under the N1-N3 treatment remained consistent in the two growth seasons. GOGAT maintained a similar pattern of change under N2-N4 from S1-S2. Taken together, these results indicate that *C. tinctoria* may have a lower demand for N during the flower bud period and early flowering period. For the S3-S4 stage, the difference in NR, NIR, GS, and GOGAT activity was significantly increased between the N1 treatment and the other N treatments. At this point, N metabolism in *C. tinctoria* under N-starvation conditions was inhibited. However, GDH activity was significantly increased under low N conditions. Both the N1 and N2 treatments resulted in values that were significantly greater than those under the normal-N-supply levels (CK) during the four harvest periods in 2020–2021 ($P < 0.05$). The GDH activities of the two growth seasons were 1489.45-2276.33 nmol min^{-1}·g^{-1} and 1422.38-2045.69 nmol min^{-1}·g^{-1}, respectively.

Correlation analysis. Correlation figures were plotted by integrating data from the four stages from 2020–2021 (Fig. 8). The leaf N content in 2020–2021 was significantly positively correlated with NR activity, NIR activity, GS activity, GOGAT activity, soluble protein content and total amino acid content but significantly negatively correlated with GDH activity ($P < 0.05$).

Discussion

Effects of reduced N fertilizer application on production and N content. N is one of the essential nutrients for plant growth and is involved in the whole process of physiology and biomass accumulation[19]. In the present study, the formation of flower buds (or flowers) was inhibited under N-starvation conditions, while slight N deficiency levels did not affect flowering numbers or leaf biomass (Fig. 2), which suggests that slight N deficiency is equally effective in regulating N assimilation. This expression pattern may be attributed to better N assimilation and photoassimilation efficiency enabling accelerated transfer of reproductive organs[20]. When NUE of plants increases, plant photosynthates can be converted into transportable substances to promote the development of reproductive structures (leaves or flowers)[21]. Furthermore, suitable low N levels may serve as signals of plant flowering in advance, promoting precocious maturation and early entry into the flowering stage[18]. Previous studies have reported similar results: lower N application did not significantly inhibit *C. tinctoria* flowering, further suggesting that a substantial increase in N concentration may not be a strategy to improve yield. As suggested in a cotton test system, the degree of reproductive organ development is closely related to leaf and physiological information[22]. N reduction treatment maintained leaf photosynthesis and assimilation product transportation, and further ensured the accumulation of mature leaf weight and total biomass of plants. In this study, the variation pattern of single-leaf weight and total functional leaf weight also provides evidence for this view (Fig. 2). Similar to the high NUE and high yield characteristics of *Brassica napus*[23] and maize[21] under low N conditions, reducing the crop N fertilizer demand is beneficial for both the environment and growers. Production differed in 2020 and 2021 (Fig. 1), which may have been attributed to higher temperature values, especially during the S3-S4 stage, resulting in both lower culture indoor humidity and accelerated shedding of the *C. tinctoria* inflorescence, thus reducing the final statistical yield. The content of N in leaves regulates physiological processes such as plant N assimilation, enzyme activity, and energy transport. Even if the reduced application of N fertilizer reduces the N content of the leaves (Fig. 3A), it does not mean that the N assimilation efficiency is inhibited, which is consistent with results reported for *Cyclocarya paliurus*[24].

Effects of reduced N fertilizer application on the chloroplast fluorescence system and photosynthetic parameters. The plant chlorophyll concentration was associated with the level of photosynthesis. It is clear that the N-starvation conditions in this article hindered the biosynthetic pathway of chlorophyll (Fig. 3), which in turn inhibited *C. tinctoria* photosynthesis. Appropriate amounts of low N treatment resulted in a chloroplast system similar to that under normal N levels, further demonstrating that the photoassimilation process in plants can proceed relatively easily under low N conditions[25], which provide evidence for the adaptation of *C. tinctoria* to a low N environment. Stomatal and nonstomatal limitations influence the photosynthetic rate. The reduction in the chloroplast system and chlorophyll fluorescence parameters under nitrate stress was due to nonstomatal restriction (Fig. 4). A decrease in intercellular CO_2 concentration and stomatal conductance under low N stress signals confirmed a lower stomatal permeability (Fig. 5), and the transpiration rate is closely related to the plant water absorption rate and stomatal opening[26] Furthermore, chlorophyll fluorescence dynamics have unique roles in the absorption, transmission, dissipation and distribution of light energy during leaf photosynthesis[27]. It was found that the plant response to stress could be rapidly expressed by chlorophyll fluorescence parameters[28].

N-starvation conditions reduce the transfer efficiency of the energy absorbed from the antenna chlorophyll a to the reaction center of PSII and/or damage or dissociate light-harvesting proteins. Analysis of the chlorophyll fluorescence parameters (Fig. 4) revealed that proper low N treatment prevents the occurrence of chronic photoinhibition by retaining the proper reduction process of the oxygen evolving complex and quinone A to maintain the energy capture efficiency [29]. However, with the degree of stress and chronic photoinhibition, the efficiency of energy capture decreases[4]. In this paper, Fv/Fm, Fv'/Fm' and qP decreased under N-starvation conditions, while qN exhibited the opposite trend, suggesting that stress suppresses photoenergy capture and electron transfer efficiencies and increases optical energy dissipation. Other abiotic stress conditions, such as salt stress or heavy metal exposure, resulted in similar findings to those obtained in the present study[30, 31].

Effects of reduced N fertilizer application on nitrogen metabolism-related enzymatic activity and amino acid accumulation. N application affects carbon assimilation, photosynthesis and N metabolism[12]. NR and NIR are major enzymes involved in N metabolism, where NR is the rate-limiting enzyme affecting

nitrate assimilation[32, 33]. NR promotes the absorption of nitrate and forms nitrate, further binding to NIR to generate ammonia[34]. In the present study, NR and NIR activity increased with N, especially during S3-S4, indicating the increasing dependence of plant growth on N fertilizer over harvest time (Fig. 7). These findings are further reinforced by the correlation between NR and NIR (Fig. 8). Multiple experimental systems support this result, including those conducted in tomato[35], cucumber[4], and soybean[14]. GS and GOGAT were analyzed simultaneously with the amino acid synthesis process (Fig. 6). GS plays an important role in the N assimilation process, but its mechanism of action is prone to change depending on the metabolic and growth conditions[36]. Further enhancement of NR and NIR activity and a continued supply of ammonium improve GS activity, a result also demonstrated in the present experiment. In addition, with the increase in N levels, the activities of GS and GOGAT significantly increased (Fig. 7), demonstrating that N metabolism in *C. tinctoria* leaves was tightly regulated by nitrate concentrations. Plants synthesize amino acids from nitrite-N after a series of enzymatic reactions through GS and GOGAT, which provides sufficient ingredients for protein synthesis[12]. It is believed that many factors are involved in affecting nitrite-N content and amino acid metabolism. In this experiment, some amino acid (e.g., arginine) levels were maintained under appropriate N fertilizer conditions, most of which met the needs of source library organs to achieve the required balance, and similar results were observed in *Ipomoea batatas*[37]. However, the decrease in amino acid content when plants were subjected to N-starvation conditions could be attributable to decreases in the protein decomposition rate or a higher rate of protein synthesis. In this study, normal N levels consistently maintained the soluble protein content, which was the result of the N assimilate (GS and GOGAT) activity supplying amino acids and binding them to a protein structural complex. The GS and GOGAT activities under low N conditions were similar to the accumulation pattern of total free amino acids, while the same accumulation patterns of protein and soluble amino acids further indicated that suitable N fertilizer reduction conditions could guarantee the normal transport of N metabolic assimilates. GPT (Glutamic-pyruvic transaminase) and GOT (Glutamic oxaloacetic transaminase) use Glu as the basic reactant for the synthesis of aspartate and glutathione, and amino acids such as aspartate and Glu are important products of N metabolism[20]. As observed in the current study, these amino acids change with changes in protein synthesis or decomposition[38]. Here, Met, Ala, Val, and aromatic amino acids (including Tyr and Phe) are the precursor substances constituting the secondary metabolites that compete with protein synthesis[39]. It is therefore not surprising that the accumulation patterns of these amino acids and proteins are similar under low N conditions. Pro, Ser, and Leu can release signaling molecules under stress conditions and subsequently undergo a substantial increase, so low N stress promotes greater accumulation of Pro, Ser and Leu in leaves (Table 1)[40, 41, 42, 43]. In a study in *Arabidopsis*, Thr and Lys were significantly increased under abiotic stress conditions[44], which is consistent with the performance of *C. tinctoria* under low N conditions. Gly under N treatment can produce free ammonium by oxidative decarboxylation, while the remaining part of the amino group is transferred to Glu to generate glutamine[45]. Therefore, Gly and Glu have opposite accumulation patterns. The guanidine group of arginine is hydrolyzed by asparaginase, glutaminase, and arginase to reactivate N, thereby affecting the storage and transport of plant N[42]. In this experiment, the Arg content was maximal under slightly low N conditions. We speculate that this specific accumulation mode may play an important role in N metabolism and maintenance of the yield of *C. tinctoria*. GDH plays a role in shunting plant carbon and N. When plants are under nutritional stress, GDH activity is greatly increased, and the carbon in amino acids is returned to carbon metabolism[30, 45]. In this study, N regulated GDH activity to effectively improve N assimilation (Fig. 7). The relative humidity during the harvest season in 2021 was relatively high, which may have affected the microclimate environment in which *C. tinctoria* grew, thereby increasing the temperature of the leaves and ultimately affecting N metabolism. Plants usually exhibit changes in physiological activities, such as photosynthesis and carbohydrate metabolism, under this type of climate change.

Chlorophyll levels in the leaf indicate the efficiency of N assimilation in plants because of the mobile nature of the N and because chlorophyll is synthesized through the N metabolism pathway. In the present study, an appropriately low N level maintained the chlorophyll level, which indicated that N metabolism in leaves was well managed and contributed to the formation of photosynthetic products[20], the absorption of nitrates, the transport of compounds to leaves, and the proper transport of proteins and amino acids[46]. Here, the rise of several amino acids (including Thr, Ser, Gly, Leu, Ile, Lys and Arg) under low N treatment is likely to provide motivation for photosynthesis of plants, thereby maintaining photosynthetic efficiency[47, 48]. In addition, appropriately low N levels may maintain the transfer of amino acid activity to leaf biomass, better facilitate N assimilation and ultimately ensure an optimal number of flowers in *C. tinctoria*.

Conclusions

To the best of our knowledge, this was the first project to study the impacts of reduced NO_3^- level on productivity, N metabolism, photosynthetic light-use efficiency in edible *Coreopsis tinctoria* grown indoor. In the present study, N fertilizer reduction improved the N assimilation levels of *C. tinctoria* and could be used to drive optimal plant NUE. Nitrogen reduction (1/8N-1/2N) did not damage the photosynthetic system and chlorophyll fluorescence system of *C. tinctoria* leaves.Suitable N fertilizer reduction maintained activities of NR, NIR, GS and GOGAT, as well as the contents of total amino acids and protein in leaves. The increase in Thr、Ser、Leu、Ile and Arg under reduced N conditions is likely to benefit leaf N storage and transport. Reducing N levels proved to be a viable management approach to balance N metabolism in *C. tinctoria* leaves to maintain promising yields in 2020 and 2021. In addition, this study also shows that 1/8 NO_3^- prior to harvest could be one of the strategies as the productivity were maintained and planting costs were reduced in *Coreopsis tinctoria*.

Materials And Methods

Experimental conditions. The experiment was carried out from March 2020 to August 2021 at the Institute of Agricultural Mechanization, Xinjiang Academy of Agricultural Sciences, Urumqi (43°82′ N, 87°59′ E), China. The recorded humidity and temperature conditions in the solar greenhouse were almost the same, while both the maximum and the minimum temperatures in 2020 were higher than those in 2021 (Fig. 1).

Plant materials and experimental design. *C. tinctoria* seeds were collected from the Ke Liyang township, Xinjiang Province, China, at the foot of the Kunlun Mountains (37°27′ N, 77°84′ E). The research group declares that the collection of *C. tinctoria* seeds to comply with national and international norms and legislation. The *C. tinctoria* seedlings were grown in substrate (vermiculite/perlite = 2/1) to the four-leaf stage and transferred to a polyethylene plastic basin filled with 8 L nutrient solution (45 cm × 31 cm × 15 cm) for a hydroponic experiment.

The stable supply of calcium ions is beneficial to the rooting and development of *C. tinctoria*, so calcium nitrate was selected as the nitrogen source in this experiment. The modified Hoagland nutrient solution comprised 0.17 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.2 mM NH_4NO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4{\cdot}7H_2O$, 2 mM $FeSO_4{\cdot}7H_2O$, 2.2 mM EDTA-2Na, 1 mM H_3BO_3, 1.48 mM $MnSO_4$, 5.3 mM $ZnSO_4$, 0.1 mM Na_2MoO_4, 0.02 mM $CuSO_4$, and 0.01 mM $CoCl_2$. Five N levels were established according to preliminary results: N1 (0 mM $Ca(NO_3)_2$, 0 N), N2 (0.625 mM $Ca(NO_3)_2$, 1/8 N), N3 (1.250 mM $Ca(NO_3)_2$, 1/4 N), N4 (2.500 mM $Ca(NO_3)_2$, 1/2 N) and CK (5.000 mM, full N). Treatment of Ca^{2+} deficiency is conducted by supplementation with $CaCl_2$. The light intensity was 620 $\mu mol{\cdot}m^2{\cdot}s^{-1}$, and the photoperiod was 12 h. The lost water was replenished every two days, the nutrient solution was replaced every five days and the pH value of the nutrient solution was maintained at 7.0 ± 0.1. When changing the nutrient solution, the roots were repeatedly rinsed with distilled water to remove residual nutrients. The four harvest periods were as follows: the budding stage (S1, 63 days after emergence (DAE)), initial flowering stage (S2, 78 DAE), full flowering stage (S3, 93 DAE) and final flowering stage (S4, 108 DAE). The third mature leaf below the shoot tip were selected for determination of N, amino acid and protein contents and enzyme activities related to N metabolism. The collected samples were stored at -80°C. Each treatment included 3 biological replicates. In addition, the number of flowers (flower buds) at different harvest stages and the single leaf biomass and functional leaf weight at full bloom were also determined[12].

Leaf nitrogen content measurement. Dried crushed leaf (After fully grinding, pass through a 0.5mm sieve) was used to quantify total N by following the micro-Kjeldahl method[48]. To digest the plant samples, finely ground powder was weighed to about 0.2 g for each plant part and then placed carefully at the bottom of a 250 mL digesting tube. Following this, 5–6 mL concentrated H_2SO_4 was added and placed overnight to homogenize the sample. After this period, 2 mL 30% H_2O_2 was added and heated the sample at 300°C for 60 min. Every 15 min interval, 3–4 drops of H_2O_2 were added to make the digestion solution clear. Continued to add H_2O_2 until the solution become clear. After this process, sample was allowed to cool at room temperature and then poured it in 50 mL volumetric flask and completed the volume up to 50 mL by using distilled water. Out of this, 1 mL of the digested liquid was taken and poured in a 10 mL tube and completed the volume up to 10 mL with distilled water. This solution was used later for the estimation of N content.

Total N contents were determined by micro-Kjeldahl method[49]by using an element analyzer (EA3000, Euro Vector, Italy). Following formula was used for calculations:

$$N\% = \frac{(V - V0) \times N \times 1.4007}{W}$$

Where,

V: Volume (mL) of standard acid required for sample. V0: Volume (mL) of acid required for blank. N: Normality of acid. 1.4007: milliequivalent weight of N × 100. W: Sample weight (g). The final N contents were expressed as $mg{\cdot}g^{-1}$ of dry weight.

Chloroplast pigment, chlorophyll fluorescence, and gas exchange parameters. The chlorophyll content was evaluated according to the method described by Hussain et al[50]. The fresh leaves (0.2 g) were soaked in 10 mL of 80% acetone overnight, after which the extract was transferred in a 25 mL volumetric flask, which was brought to volume with distilled water. The absorbance was recorded at 663 nm, 645 nm and 470 nm, and then the contents of chlorophyll a, chlorophyll b and carotenoids were calculated. Chlorophyll fluorescence was measured by using a pulsed chlorophyll fluorometer (FMS2, Hansatech company, England). After 25 min of dark adaptation, the maximum photochemical quantum yield (Fv/Fm) of PSII in the leaves was measured. Then, the effective photochemical quantum yield of PSII (Fv'/Fm'), nonphotochemical quenching (qN) and coefficient of photochemical quenching (qP) were read after measuring light-adapted leaves. The net photosynthesis rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) of *C. tinctoria* leaves were measured from 9:30 am to 11:30 am when the samples were harvested. An LI-6400XT photosynthetic device (with the third mature leaflet from the top to the bottom of the plant) was used for photosynthesis determination. The illumination intensity of all treatments was set to 1000 μmol photons $m^2{\cdot}s^{-1}$, and the constant gas flow rate was 500 $\mu mol{\cdot}s^{-1}$. The concentration of cuvette CO_2 was set at 400 μmol CO_2 mol^{-1} air, and the chamber temperature was maintained at 28°C.

Free amino acid and protein contents. The calculation of total free amino acid content refers to[51]method. Fresh leaves (0.1 g) were homogenized by using phosphate buffer (pH 7) and the residue was filtered. In a 25 mL test tube, 1mL of extract, 1mL of 10% pyridine, and 1 mL of 2% ninhydrin solution (2.0 g ninhydrin in 100 mL distilled water) were added and heated in 100°C water for 30 min. The volume of each tube was replenished to 50 mL with distilled water, and the absorbance was recorded at 570 nm. The standard curve of L-leucine was drawn to calculate the amino acid content.

The leaves were thoroughly dried and crushed and then added to an anaerobic tube containing 15 mL of 6 $mol{\cdot}L^{-1}$ hydrochloric acid and 2.0 mL of phenol. Then, refrigerant was added to the tube, after which it was incubated at room temperature for 5 min. The anaerobic tube was evacuated and filled with high-purity N, and the tube was kept airtight. The anaerobic tube was placed in a 110°C drying tank for 22 h and then allowed to cool to room temperature. Ultrapure water was used to clean the hydrolysis tube repeatedly, and then the solution was transferred to a 50-mL volumetric flask, which was subsequently brought to volume. The hydrolysis tube was repeatedly cleaned with ultrapure water, and then the solution was transferred to a 50-mL volumetric flask and replenished to the mark. One milliliter of filtrate was repeatedly dissolved in ultrapure water in a vacuum dryer at 50°C. Finally, 1 mL of pH-2.2 buffer was added to dissolve the sample, and the free amino acid contents were determined using an automatic amino acid analyzer (Sykam S4SSD, Germany).

Determination of the soluble protein content was performed according to the method of Bradford[52]. Fresh leaves (0.2 g) were ground into a powder, fully homogenized in a solution consisting of 10 mL of 1% polyvinylpyrrolidone (pH 7.8), 1 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid, after which the mixture was finally centrifuged at 8, 000 rpm for 10 min. Bovine protein was used to generate a protein standard curve, and then the absorbance of the solution at 595 nm was recorded.

Nitrogen metabolism-related enzyme activity. Enzyme activities related to N metabolism were measured using an ELISA kit purchased from Suzhou Comin Biotechnology, Suzhou, China. A total of 1.0 g of fresh sample was fully homogenized in liquid N. Then, 1.0 g of extract and 2 mL of extraction buffer (purchased from a commercial company) were completely mixed and added to a test tube. All the test procedures were performed in accordance with the product instructions. The nitrate reductase (NR) (EC 1.7.1.3), nitrite reductase (NIR) (EC 1.7.1.4) and GS (glutamine synthetase) (EC 6.3.1.2) activities were recorded at 540 nm, while the glutamate synthetase (GOGAT) (EC 1.4.7.1) and glutamate dehydrogenase (GDH) (EC 1.4.1.2) activities were measured at 340 nm on a xMark™ Spectrophotometer (BIO-RAD, USA). The NR activity was expressed as 1 μmol NO_2^- production in 1 min in a 1.0 g fresh sample. The reduction of 1 μmol NO_2^- of 1.0 g of fresh leaves in 1 min was taken as one unit of NIR activity, and the absorption change of 1.0 g sample in 1 mL of reaction solution per min was considered one unit of GS activity. The GOGAT activity was defined as the oxidation of 1 nmol NADH in 1.0 g of fresh sample in 1 min. GDH activity was expressed as 1 nmol NADH reduction per minute in 1.0 g of leaves[53].

Declarations

Data Analysis. The data obtained were arranged and statistically analyzed in Microsoft Excel 2019. Tukey's (HSD) test was used to analyze the significance of different N levels at a probability level of 5% ($P < 0.05$). A graph of the test data was created using SigmaPlot 12.5 software (Systat Software, Inc., San Jose, CA, USA). The data correlations were calculated in R software package 3.4.1.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors Contributions. Zhiyuan Li and Yong Qin initiated and designed the study, Hong Jiang, Yumiti Yusupu, Lifang Zhang and Xiumei Jiang participated in the experiment, Zhiyuan Li analyzed the experimental data and wrote the manuscript, and Yong Qin and Hong Jiang provided experimental suggestions and edited the manuscript.

Data Availability Statement. The data are contained within the article and supplementary material.

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Figures

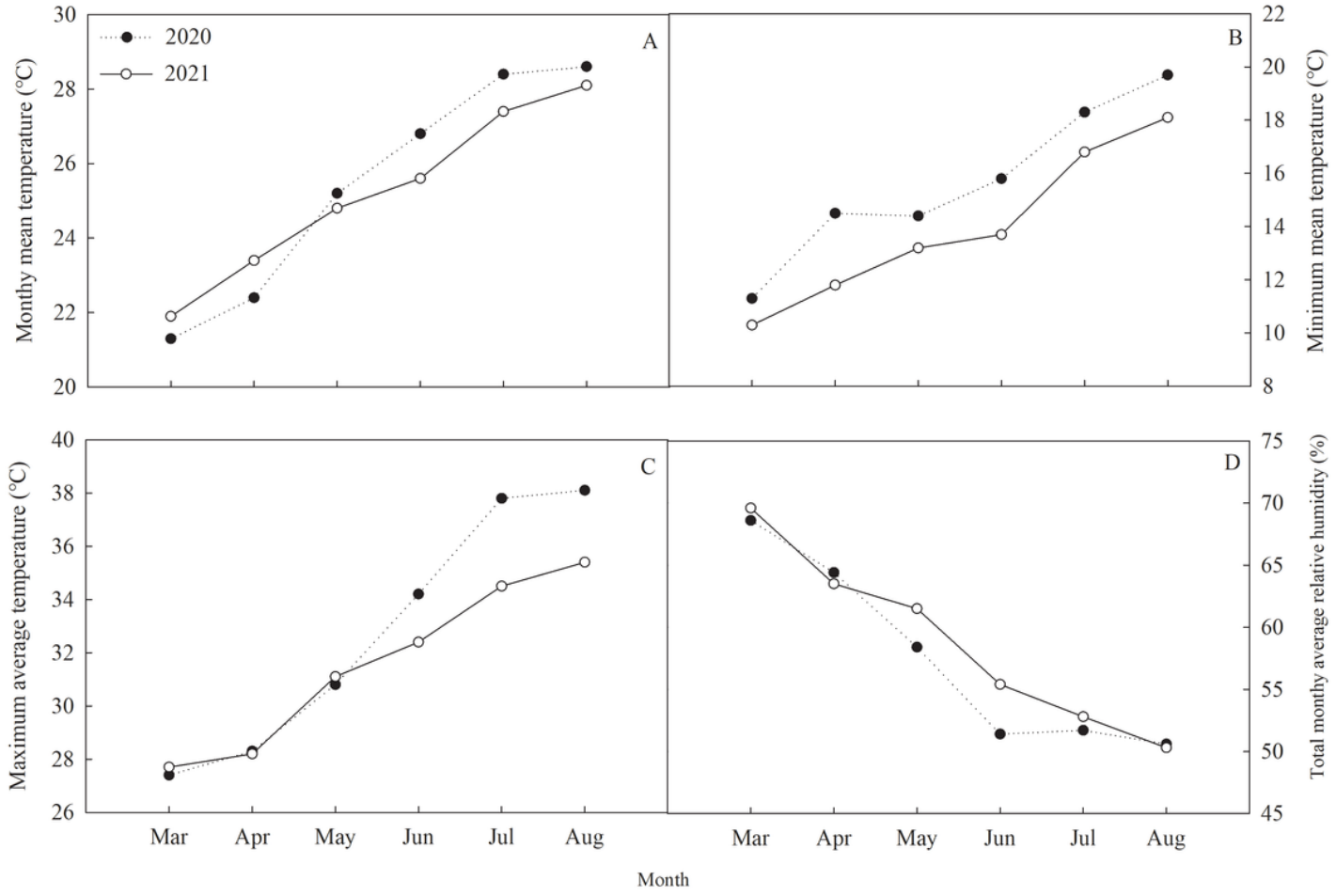

Figure 1

Data on weather parameters; minimum mean temperature, maximum mean temperature, monthly mean temperature and total monthly rainfall, during the 2020 and 2021 growth seasons.

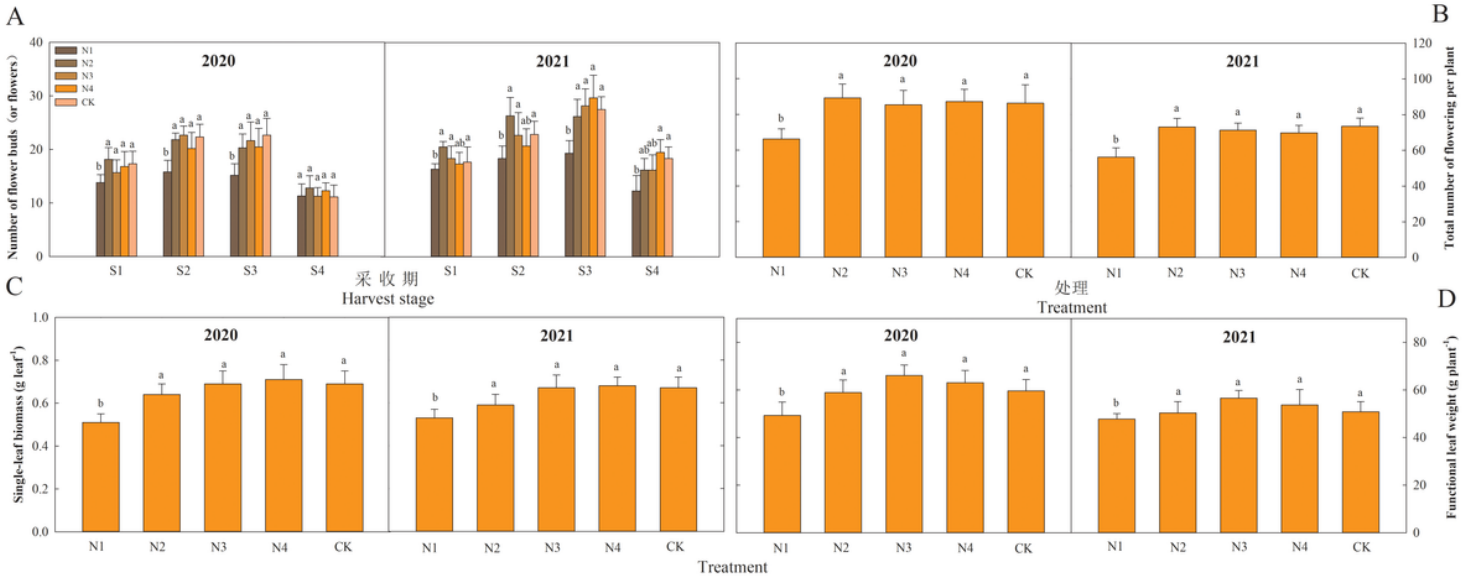

Figure 2

Effect of N treatment on number of flower buds (or flowers), total number of flowering per plant, single-leaf biomass and functional leaf weight, during the 2020 and 2021 growth seasons.

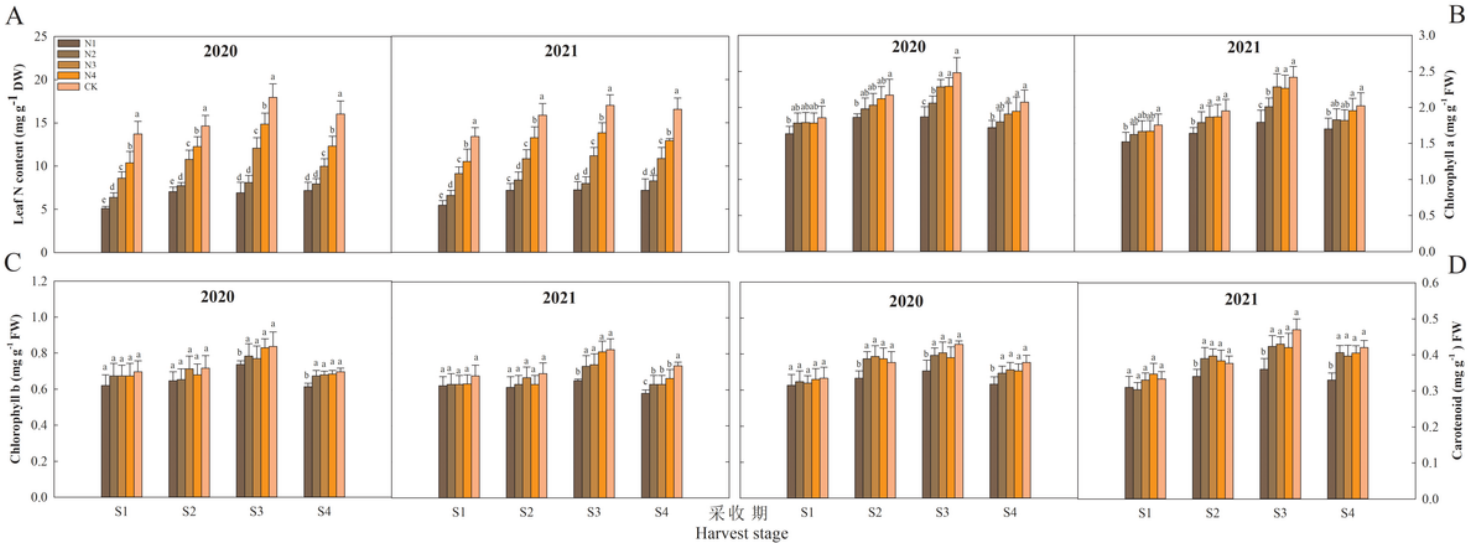

Figure 3

Effect of N treatment on leaf N concentration and chlorophyll content at S1 (bud stage), S2 (initial flowering stage), S3 (full flowering stage) and S4 (final flowering stage), during the 2020 and 2021 growth seasons, where, N1, N2, N3, N4 and N5 are 0 mM $Ca(NO_3)_2$, 0.625 mM $Ca(NO_3)_2$, 1.250 mM $Ca(NO_3)_2$, 2.500 mM $Ca(NO_3)_2$, and 5.000 mM $Ca(NO_3)_2$ respectively. Error on the bars shows standard error means.

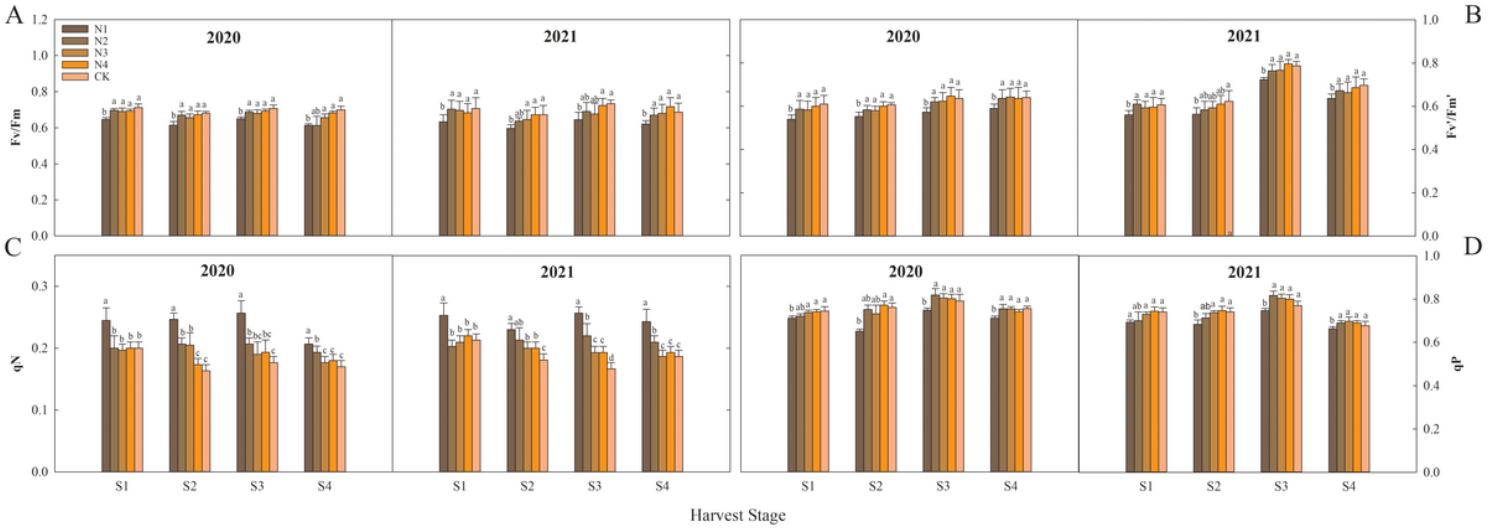

Figure 4

Effect of N treatment on chlorophyll fluorescence parameters (Fv/Fm, Fv'/Fm', qN and qP) at S1 (bud stage), S2 (initial flowering stage), S3 (full flowering stage) and S4 (final flowering stage), during the 2020 and 2021 growth seasons, where, N1, N2, N3, N4 and N5 are 0 mM $Ca(NO_3)_2$, 0.625 mM $Ca(NO_3)_2$, 1.250 mM $Ca(NO_3)_2$, 2.500 mM $Ca(NO_3)_2$, and 5.000 mM $Ca(NO_3)_2$ respectively. Error on the bars shows standard error means.

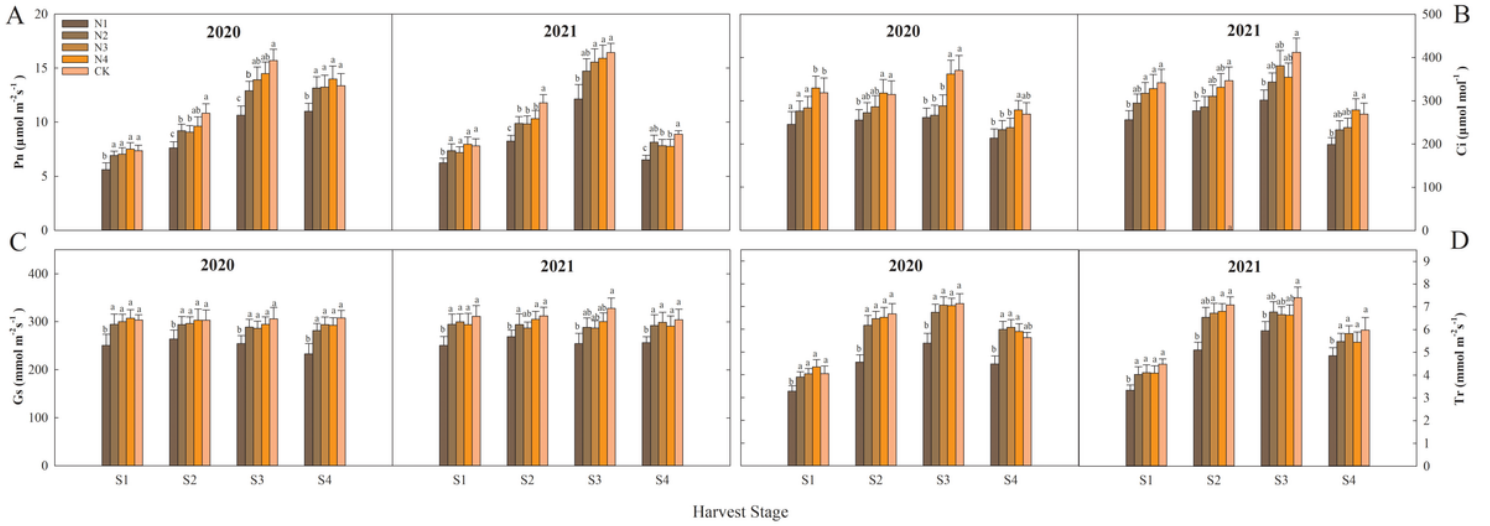

Figure 5

Effect of N treatment on photosynthetic parameters (Pn, Ci, Gs and Tr) at S1 (bud stage), S2 (initial flowering stage), S3 (full flowering stage) and S4 (final flowering stage), during the 2020 and 2021 growth seasons, where, N1, N2, N3, N4 and N5 are 0 mM $Ca(NO_3)_2$, 0.625 mM $Ca(NO_3)_2$, 1.250 mM $Ca(NO_3)_2$, 2.500 mM $Ca(NO_3)_2$, and 5.000 mM $Ca(NO_3)_2$ respectively. Error on the bars shows standard error means.

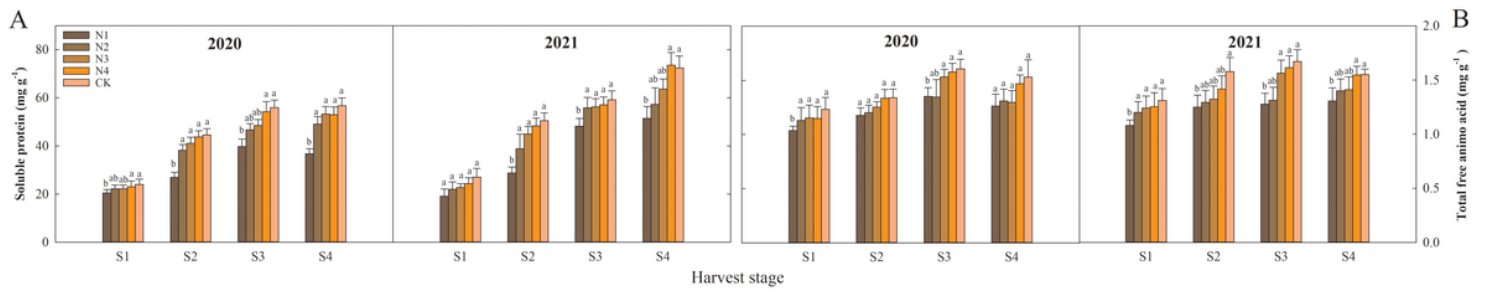

Figure 6

Effect of N treatment on soluble protein and amino acid at S1 (bud stage), S2 (initial flowering stage), S3 (full flowering stage) and S4 (final flowering stage), during the 2020 and 2021 growth seasons, where, N1, N2, N3, N4 and N5 are 0 mM $Ca(NO_3)_2$, 0.625 mM $Ca(NO_3)_2$, 1.250 mM $Ca(NO_3)_2$, 2.500 mM $Ca(NO_3)_2$, and 5.000 mM $Ca(NO_3)_2$ respectively. Error on the bars shows standard error means.

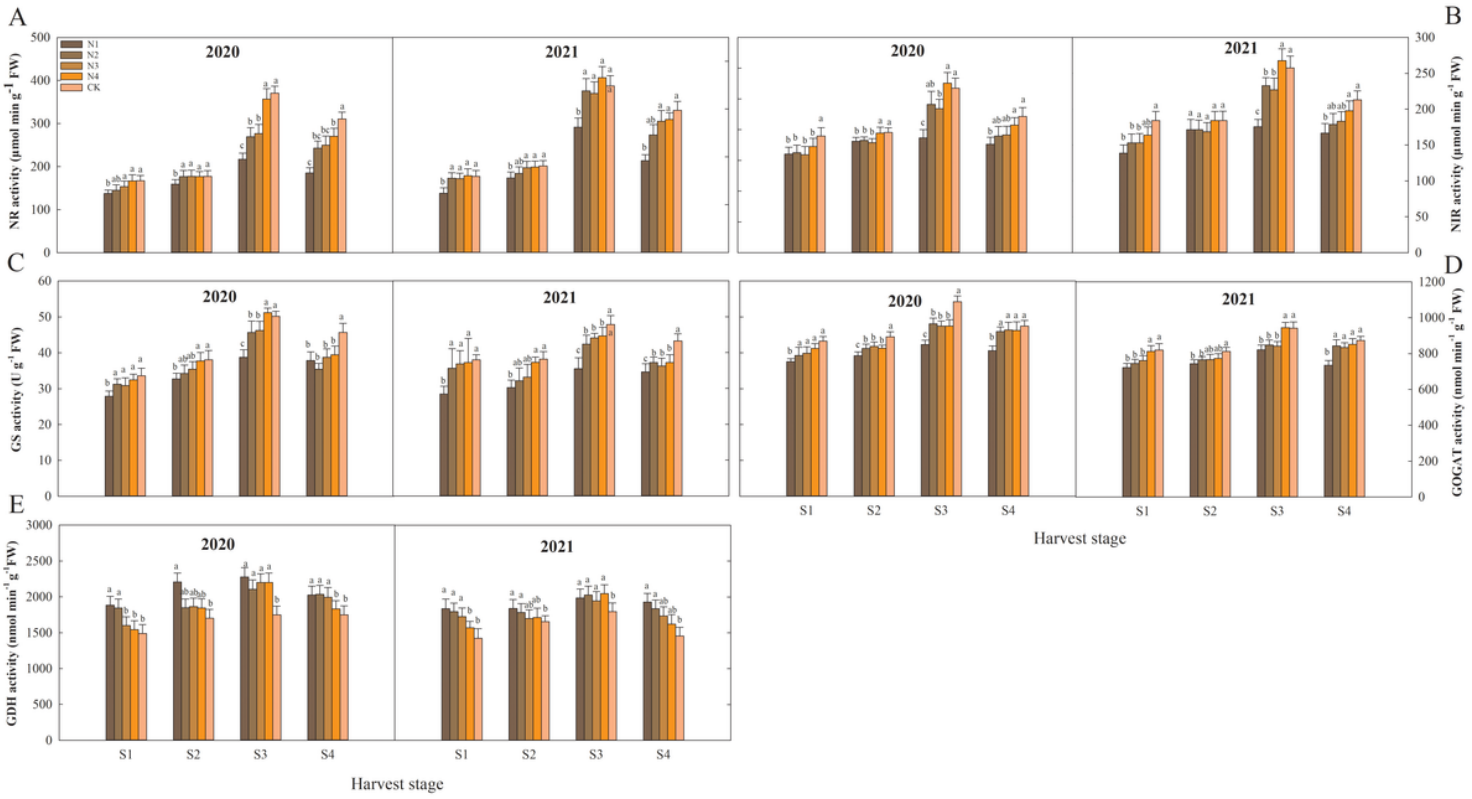

Figure 7

Effect of N treatment on nitrogen metabolism related-enzyme at S1 (bud stage), S2 (initial flowering stage), S3 (full flowering stage) and S4 (final flowering stage), during the 2020 and 2021 growth seasons, where, N1, N2, N3, N4 and N5 are 0 mM $Ca(NO_3)_2$, 0.625 mM $Ca(NO_3)_2$, 1.250 mM $Ca(NO_3)_2$, 2.500 mM $Ca(NO_3)_2$, and 5.000 mM $Ca(NO_3)_2$, respectively. Error on the bars shows standard error means.

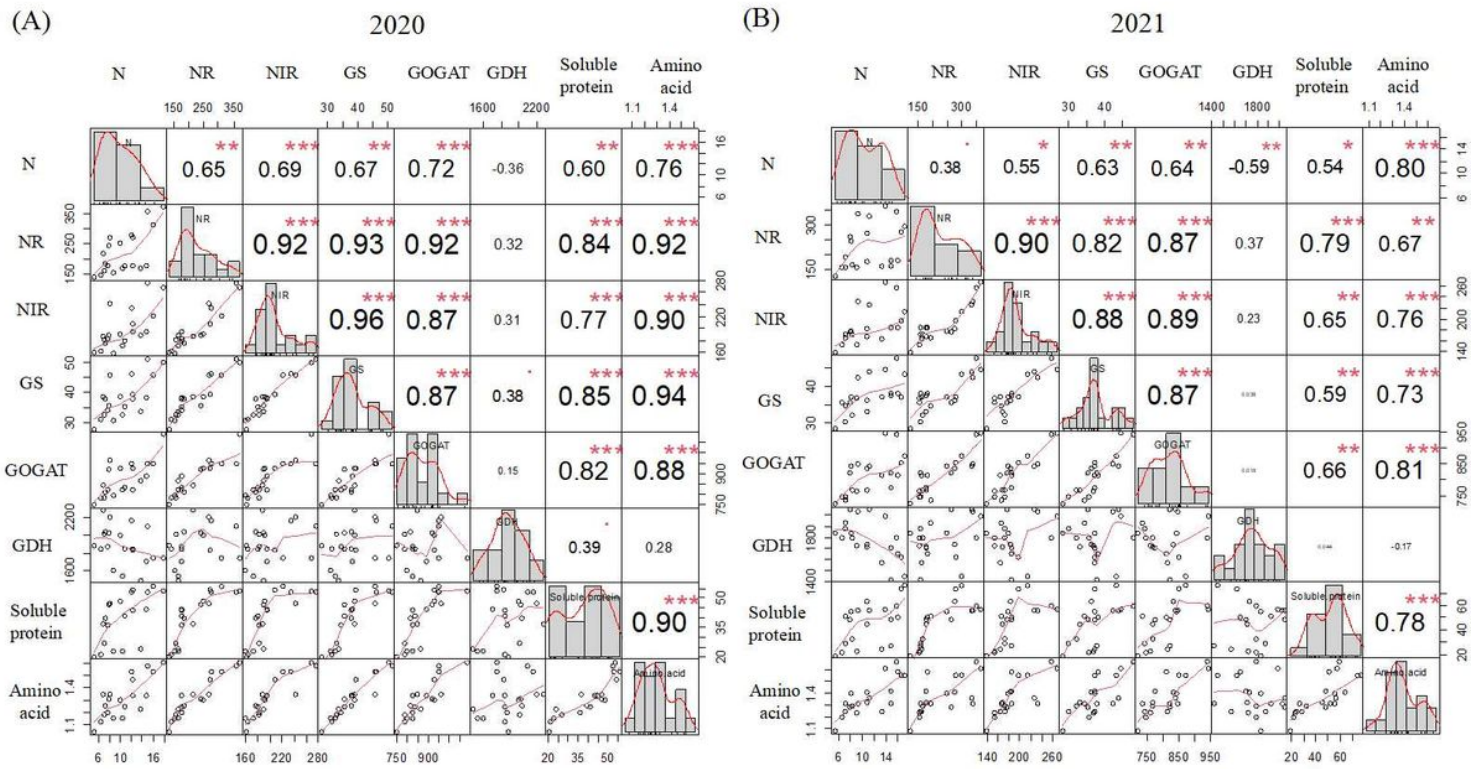

Figure 8

Relationship between leaf N content and peak activities of N metabolic enzymes (NR, NIR, GS, GOGAT, GDH), soluble protein and amino acid of *C. tinctoria* during the A) 2020 and B) 2021 harvesting seasons. The bars and line, shown with observation are regarded as correlation coefficients as 1 (r = 1). Other line graphs and correlation coefficients represent the comparative values to r =1. Where * = significant ($P \leq 0.05$), **= highly significant ($P \leq 0.01$), and *** = very highly significant ($P \leq 0.001$).