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A Maize Mutant Impaired in SL Biosynthesis (*zmccd8*) Shows a Lower Growth, an Altered Response to Nitrogen Starvation, and a Potential Secondary Effect on Drought Tolerance

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Abstract: Strigolactones (SLs) are essential phytohormones involved in plant development and interaction with the rhizosphere, regulating shoot branching, root architecture, and leaf senescence for nutrient reallocation. The *Zea mays* L. *zmccd8* mutant, defective in SL biosynthesis, shows various architectural changes and reduced growth. This study investigates *zmccd8* and wild-type (WT) maize plants under two nutritional treatments (N-shortage vs. N-provision as urea). Morphometric analysis, chlorophyll and anthocyanin indexes, drought-related parameters, and gene expression were measured at specific time points. The *zmccd8* mutant displayed reduced growth, such as shorter stems, fewer leaves, and lower kernel yield, regardless of the nutritional regime, confirming the crucial role of SLs. Additionally, *zmccd8* plants exhibited lower chlorophyll content, particularly under N-deprivation, indicating SL necessity for proper senescence and nutrient mobilization. Increased anthocyanin accumulation in *zmccd8* under N-shortage suggested a stress mitigation attempt, unlike WT plants. Furthermore, *zmccd8* plants showed signs of increased water stress, likely due to impaired stomatal regulation, highlighting SLs role in drought tolerance. Molecular analysis confirmed higher expression of SL biosynthesis genes in WT under N-shortage, while *zmccd8* lacked this response. These findings underscore SL importance in maize growth, stress responses, and nutrient allocation, suggesting potential agricultural applications for enhancing crop resilience.

Keywords: chlorophyll; drought tolerance; gene expression; growth; nitrogen; remobilization; strigolactones; *Zea mays* L.; *zmccd8* mutant

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1. Introduction

Nitrogen (N) is the mineral nutrient required in the greatest amount for plant growth and crop productivity, being a crucial component of proteins, amino acids, nucleic acids (DNA, RNA), membrane lipids, ATP, NADH/NADPH, co-enzymes, photo-synthetic pigments (chlorophyll), phytohormones (cytokinins and auxin), secondary metabolites (alkaloids), and other important molecules [1]. In addition to its crucial role as a nutrient, N is also involved in several abiotic stress responses, such as drought, salt stress, and deficiencies of other macro- and micronutrients [2] and plays essential roles in various developmental processes, including growth, leaf area expansion, and the production of biomass [3].

In agricultural soils, N predominantly exists in inorganic forms, such as nitrate (NO_3^-) and ammonium (NH_4^+), while organic forms, such as urea, free amino acids, and short peptides, are more relevant in extremely N-poor and cold ecosystems [4]. Soil urea can be quickly hydrolysed to NH_4^+ by ureases, which is in turn rapidly converted into NO_3^- [5,6]. Since N acts not only as a nutrient but also as a regulatory signal, total N availability

and the forms supplied affect seed germination, plant growth, root and leaf functionalities, hormonal balance, and seed production [7]. In particular, the major adaptations to N availability consist of the changes in uptake activity and in the modulation of the root system architecture (RSA) [8]. Interestingly, N deficiency induces a stress condition visible as growth reduction and leaf chlorosis, to which the plant responds with several compensation responses. These responses are in turn regulated by biochemical and molecular adjustments, among which the induction, production, and exudation of strigolactone (SL) [9–13]. In addition to N, phosphate (P) and sulphur (S) deficiencies are also powerful inducers of the biosynthesis and exudation of SLs [14,15].

SLs are a class of terpenoid lactones derived from carotenoids, widely acknowledged as a novel group of plant hormones that play critical roles in various aspects of plant development and growth, such as shoot and root architecture [16–20], the development of flowers [21], leaf senescence [22], and photomorphogenesis [23].

The employment of SL mutants is a useful tool to characterise their physiological roles. Guan and colleagues [24] identified a maize mutant (*zmccd8*) with a short mild branching phenotype and a smaller root system that was unable to synthesise SLs due to the insertion of a *Dissociation* (*Ds*) transposon in the third exon of the *ZmCCD8* gene that is required for the biosynthesis of SLs. In a previous study, the *zmccd8* mutant was used to provide new knowledge on the SL-mediated molecular regulation of maize acclimation to N fluctuations, highlighting an association with changes in the content and distribution of S and iron (Fe) [20].

In the present study, the same *zmccd8* mutant was employed to distinguish the general effects of SLs, such as those on plant development, from those implicated in the response to N. Maize plants of wild-type (WT) and *zmccd8* mutant genotypes (both in a B73 background) were grown in an open field and subjected to two different N-nutritional regimes. The objective was to examine the influence of SLs on maize growth and stress responses under N deficiency. Specifically, this study aimed to determine how SLs affect plant architecture, chlorophyll and anthocyanin contents, and water stress tolerance in both WT and *zmccd8* mutant maize plants. By comparing the responses of these plants to different nutritional treatments, we evaluated the role of SLs in regulating nutrient allocation and stress adaptation mechanisms. Morphometric analysis, chlorophyll and anthocyanin indexes, transpiration rate, stomatal conductance, and photosystem II efficiency, together with the expression of some selected genes, were determined at specific time points. Our findings showed that, during N-starvation, the production of SLs is critical for the induction of senescence, promoting resource reallocation to younger tissues—a typical mechanism of tolerance to low N. Moreover, the present results led us to also hypothesise a correlation between -N-induced SL production and improved water stress tolerance.

2. Results

2.1. Phenotypic Analysis of Growth Traits

In order to observe differences between (wild-type) WT and *zmccd8* mutant plants, several phenotypical analyses were performed on different days after the sowing (DAS) (Figure 1). Considering the stem height (Figure 1A), no significant difference between WT and *zmccd8* was detected at 38 DAS, while, starting from 45 DAS, the WT plants appeared significantly taller than *zmccd8*, reaching values 50% higher in the WT with respect to *zmccd8* at 60 DAS. After the provision of urea as an N source at 58 DAS, no significant differences were observed between WT and *zmccd8* plants treated with N compared to the same genotype that was untreated. The greatest difference among genotypes was detected at 68 DAS, in correspondence of which both WT +N and WT -N were around 40 cm higher (+56%) than *zmccd8* -N and *zmccd8* +N, respectively. This trend was observed also at 81 and 89 DAS, even though the differences were smaller (+20% in the WT compared to *zmccd8*).

Regarding the number of total leaves (Figure 1B), no significant difference between WT and *zmccd8* was detected at 38 DAS, while, from 45 DAS, the WT plants started to display a significantly higher leaf number than *zmccd8* (+10%). A peak in leaf number was

observed in both WT and *zmccd8* at 68 DAS, but no significant differences were observed in response to urea in both genotypes. After 68 DAS, the number of leaves remained constant for both of them, with an average of 12 leaves for WT and 10 for *zmccd8*.

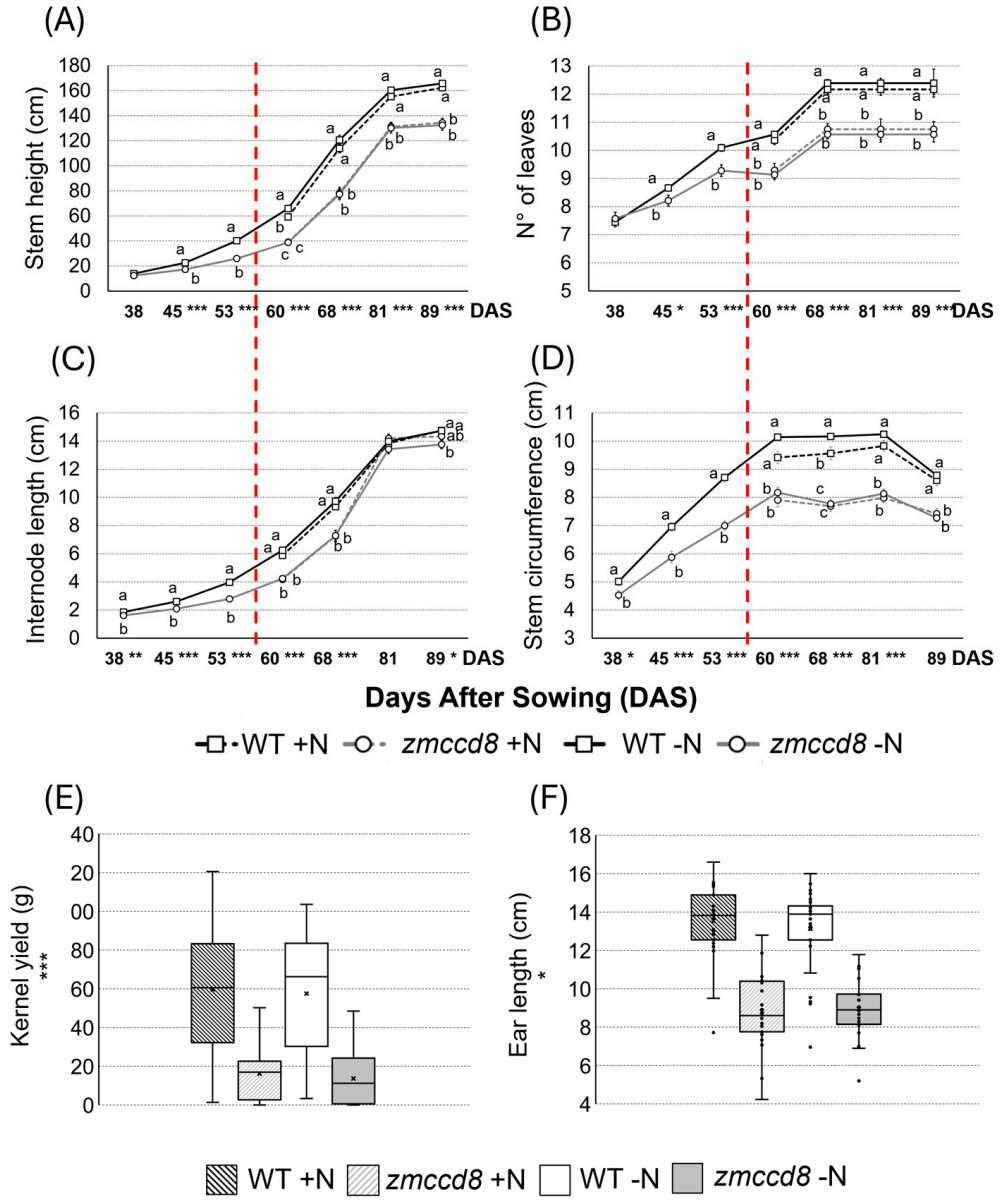

Figure 1. Phenotypic analysis of stem height (**A**), leaf number (**B**), internode length (**C**), stem circumference (**D**), kernel weight (**E**), and ears length (**F**) for wild-type (WT) and *zmccd8* mutant plants at different days after sowing (DAS) under two N treatments. Error bars represent the mean ± SE (n = 24). At 58 DAS, urea was provided as the N source (dashed red line). Different letters indicate significant differences (at $p < 0.05$ according to LSD test) at each DAS. Based on ANOVA, the significance of F values was reported as follows: '***' $p < 0.001$; '**' $p < 0.01$; '*' $p < 0.05$; no asterisks $p > 0.05$.

Regarding the internode length (Figure 1C), differently from what was observed in the previous parameters, significant differences were detected between WT and *zmccd8* at 38 DAS, with WT displaying consistently longer internodes. However, the differences became non-significant between all of the four G x N (genotype x nitrogen treatments) at 81 DAS and turned back slightly, but were still significantly different at 89 DAS, with the WT having the longest internodes, followed by *zmccd8*. Again, no differences were observed in the genotypes in response to urea provision.

Regarding the stem circumference (Figure 1D), similarly to the internode length, significant differences between WT and *zmccd8* were detected at 38 DAS, with WT displaying a ticker stem. In the following time points, the trend remained similar. Both genotypes showed the highest stem circumference at 60 DAS, with around 10 cm for WT and 7 cm for *zmccd8*, which remained stable until 81 DAS, while at 89 DAS, a decrease in both was observed. No effects were detected in response to N provision as urea, neither in WT nor in *zmccd8*.

The results for kernel weight (Figure 1E) and ear lengths (Figure 1F) showed that, regardless of the nutritional regime, *zmccd8* was deeply impaired in both the production of adequate ears and in kernel yield.

Globally, these results showed that the *zmccd8* mutant was impaired in growth if compared to WT, but no significant changes were observed after urea provision, neither in WT nor in *zmccd8.*

2.2. Assessment of Chlorophyl and Anthocyanin Contents in Leaves

Chlorophyl (CHL) and anthocyanin (ANTH) contents were evaluated in each leaf of WT and *zmccd8* maize plants at different time points after sowing (DAS), with leaf 1 being the closest to the ground, leaf 2 immediately above, and so on until leaf 11. Therefore, leaves 9 to 11 were the youngest. Data are shown for the groups of leaves (L1-2, L3-4-5, L6-7-8, and L9-10-11), grouped according to their similar behaviours in pigment content (Figure 2). An increasing trend in CHL was observed over time (Figure 2A). WT consistently showed higher CHL levels than *zmccd8*, particularly from 60 DAS in L1-2 (+20%), and as early as 38 DAS in L3-4-5 and L6-7-8 (+45%). In younger leaves (L6-7-8), WT plants exhibited significantly higher CHL levels from the beginning of the experiment, independently from fertilisation, thus suggesting that *zmccd8* may have difficulty remobilising N to young leaves. Following urea provision (58 DAS), WT maintained consistent CHL levels even under N deficiency (-N) in young leaves (L9-10-11), whereas *zmccd8* showed a small but significant decrease in CHL value (−10%) compared to those plants fertilised with N (*zmccd8* +N), a difference that remained constant at 81 and 89 DAS. In older leaves, no differences between genotypes were observed.

Anthocyanin (ANTH) content generally showed a reduction over the duration of the analysis across all leaf groups (Figure 2B). ANTH values in all leaf groups were always significantly higher in *zmccd8* than in WT by an average of +30%. Comparing plants grown with or without urea, no significant differences in ANTH content were observed within the same genotype in L1-2, L3-4-5, and L6-7-8. However, in L9-10-11, *zmccd8* plants subjected to continuous N-deficiency (*zmccd8* -N) exhibited a slight but significant increase (+8%) in ANTH content compared to *zmccd8* plants treated with urea (*zmccd8* +N) at 68 and 81 DAS, corresponding to 10 and 23 days from urea treatment. Conversely, WT showed no significant differences in response to fertilization. These results indicated that *zmccd8* generally exhibited higher ANTH levels than WT and that N deficiency induced ANTH accumulation in *zmccd8*, while having no effect on ANTH levels in the WT.

2.3. Assessment of Stomatal Conductance, Transpiration Rate, and Photosystem II Efficiency

WT and *zmccd8* stomatal conductance (*gsw*), the leaf transpiration rate (*E-app*), and Photosystem II (PSII) efficiency were measured at 38, 45, and 53 DAS and at 60, 68, 81, and 89 DAS (both in -N and urea supplied plants). As for the CHL and ANTH content, every leaf was analysed, and the data were assembled in four different groups based on the similarities of their behaviour. The results shown in Figure 3 focus on the youngest leaves (9 to 11), which were particularly responsive to variations in N status. Complete results for every leaf are reported in Figure S1.

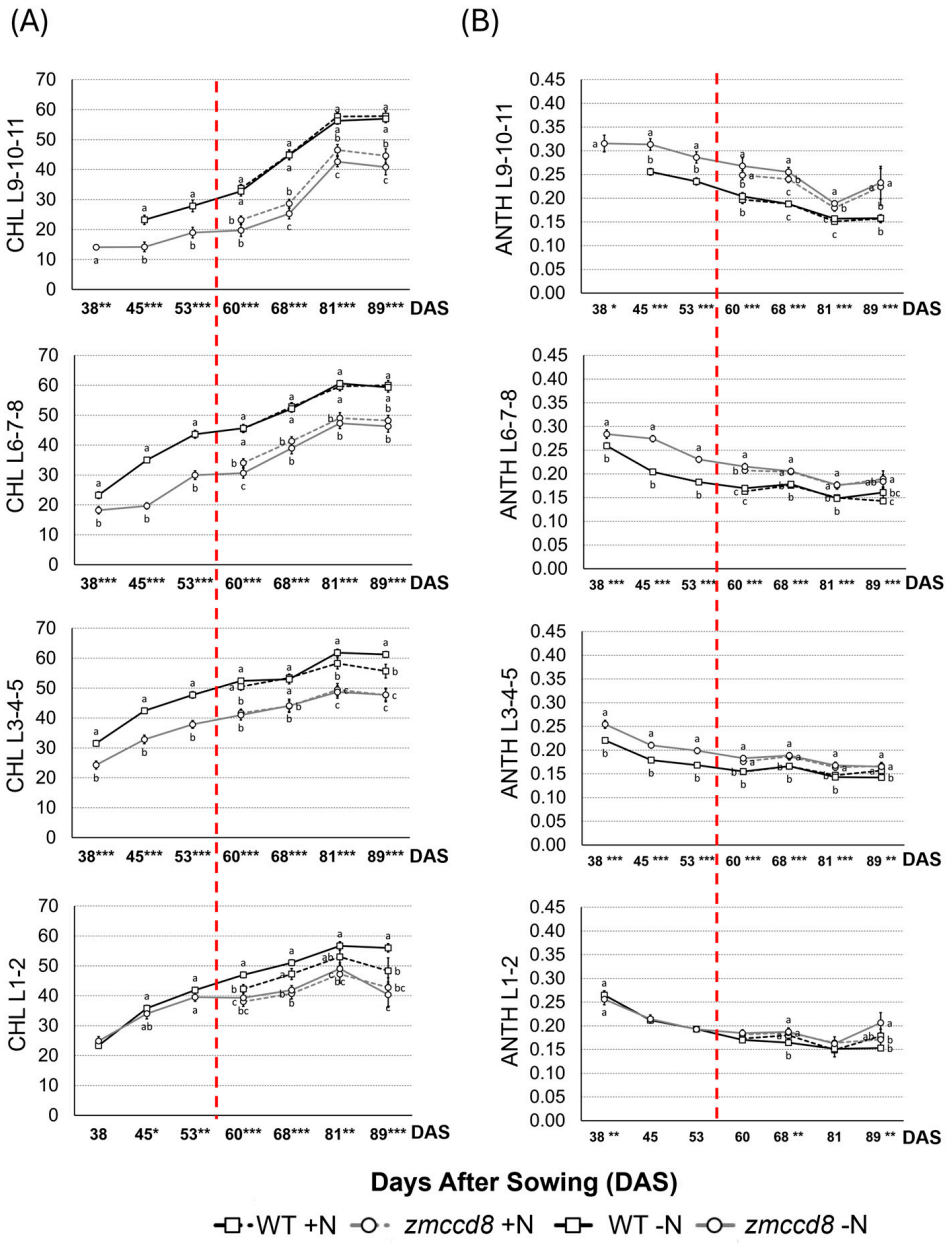

Figure 2. Profiles in chlorophyll content (**A**) and anthocyanin levels (**B**) in four different groups of maize leaves (L1-2; L3-4-5; L6-7-8; L9-10-11). Error bars represent the mean of six biological replicates ± SE. At 58 DAS, urea was provided as the N source (dashed red line). Different letters indicate significant differences (at $p < 0.05$ according to LSD test) at each DAS. Based on ANOVA, the significance of F values was reported as follows: '***' $p < 0.001$; '**' $p < 0.01$; '*' $p < 0.05$; no asterisks $p > 0.05$.

The maximum levels of *gsw* and *E-app* were observed at 60 DAS, which occurred a few days after N-fertilisation with urea in all leaf groups. Up to 68 DAS, the behaviour of the two genotypes was highly similar. However, at 81 DAS, substantial differences begin to emerge; specifically, a significant reduction in *gsw* was observed in WT plants grown without N (-N) when compared to the mutant (Figure 3A). Similarly, a significant reduction in the leaf transpiration rate (Figure 3B) and PSII efficiency (Figure 3C) was observed in WT plants subjected to N-deficiency, while no alterations in these parameters were noted in *zmccd8* plants under the same conditions. Photosystem II efficiency increased in both genotypes until 81 DAS (Figure 3C). While these levels remained constant at 89 DAS in the WT, they decreased in the *zmccd8*. In this case, the behaviour of the two genotypes was not affected by N-fertilisation. These results indicate that *zmccd8* is impaired in its

ability to implement a secondary mechanism of protection from drought stress in response to N-deficiency, leading us to hypothesise the existence of a crosstalk between N-starvation and water stress response in maize that might depend on SLs.

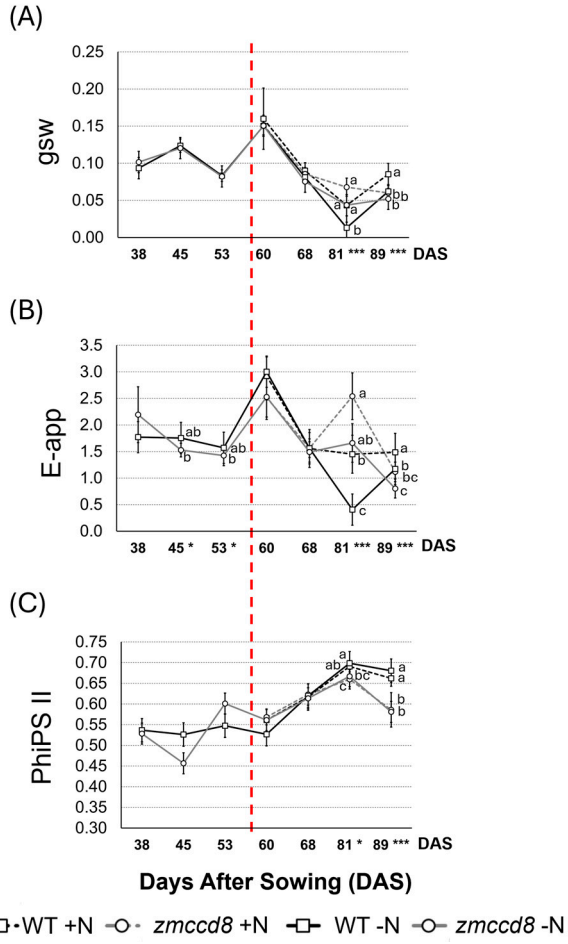

Figure 3. Profiles of stomatal conductance (gsw, mol H_2O $m^{-2}s^{-1}$) (**A**), leaf transpiration (E-app, mol H_2O $m^{-2}s^{-1}$) (**B**), and photosystem II efficiency (PhiPS II) (**C**) in the group of leaves 9-10-11. Error bars represent the mean of six biological replicates ± SE. At 58 DAS, urea was provided as the N source (dashed red line). Different letters indicate significant differences (at $p < 0.05$ according to LSD test) at each DAS. Based on ANOVA, the significance of F values was reported as follows: '***' $p < 0.001$; '*' $p < 0.05$; no asterisks $p > 0.05$.

2.4. Molecular Analysis of Maize Gene Related to SL Biosynthesis and Signaling and Water Stress Response

The expression of two genes involved in SL biosynthesis (*CCD7*, *CCD8*) and one gene involved in SL signalling (*MAX2*) was analysed in the WT and *zmccd8* leaf samples of plants supplied or not supplied with urea after 10, 23, and 31 days, corresponding to 68, 81, and 89 DAS, respectively (Figure 4). As expected, *CCD8* displayed no expression in the *zmccd8* samples. After 10 days (68 DAS), no significant differences were observed in response to nutritional conditions (-N vs. urea) or between genotypes. After 23 days (81 DAS), a clear upregulation of the *CCD7* expression in both genotypes and of *CCD8* expression only in the WT in response to N-deficiency was observed. *MAX2* showed no significant variations in its expression at 81 DAS compared to 68 DAS. No statistical differences were detected in the expression of the three genes at 89 DAS (31 days after urea supply), between genotypes, or between nutritional treatments.

As far as *SULTR6* was concerned, its transcription was stimulated by N-starved WT plants at 81 DAS, while for *zmccd8*, no significant differences in response to N were mea-

sured, supporting the above-hypothesised SL-dependent crosstalk between N-starvation and water stress response in maize.

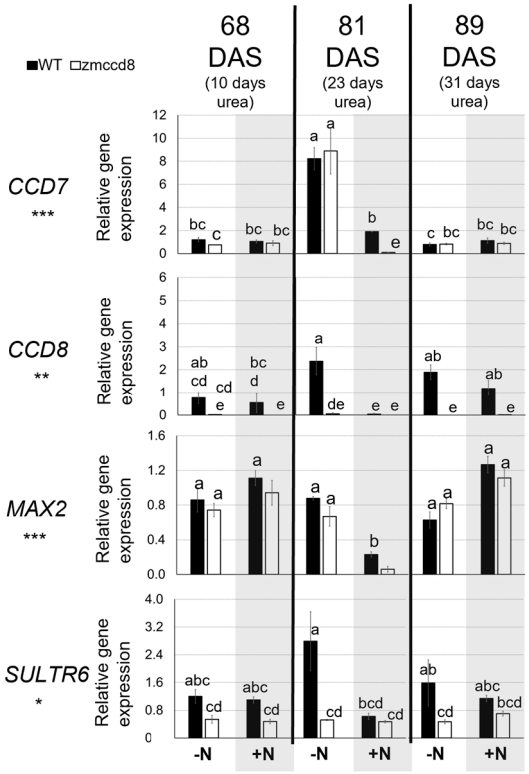

Figure 4. Relative gene expression of three genes involved in SL biosynthesis (*CCD7*, *CCD8*), signalling (*MAX2*), and drought stress (*SULTR6*) in leaf samples at three different days after sowing (DAS). Data are means ± SE for three biological replicates. Different letters indicate significant differences (at $p < 0.05$ according to LSD test) at each DAS. Based on ANOVA, the significance of F values was reported as follows: '***' $p < 0.001$; '**' $p < 0.01$; '*' $p < 0.05$; no asterisks $p > 0.05$.

3. Discussion

Strigolactones (SLs) are fundamental phytohormones acting as messengers within plants and between plants and the rhizosphere [25]. SLs are involved in many roles in plant development, including shaping the plant architecture through the inhibition of shoot branching [26] and the regulation of the root architecture [27], but they are also involved in abscisic acid, ethylene, jasmonic acid, and salicylic acid in the induction of leaf senescence to regulate the reallocation of nutrients from old to new developing tissues [28].

The maize *zmccd8::Ds* mutant is impaired in SL biosynthesis due to a knockout mutation in an essential SL biosynthetic gene that encodes the CAROTENOID CLEAVAGE DIOXYGENASE 8 (CCD8) and exhibits pleiotropic effects on maize architecture, especially regarding apical dominance and bud outgrowth, with a prominent reduction in stem diameter and internodes length [24].

In the present research, *zmccd8* mutant and WT maize plants were grown in field conditions for over 80 days and subjected to two different nutritional treatments (N-shortage vs. N-provision as urea). The *zmccd8* mutant showed an overall reduction in all the parameters analysed compared to WT, such as a shorter stem height, a lower number of leaves, a shorter internode length, a thinner stem circumference, and impaired kernel yield regardless of the nutritional regime, confirming the unequivocal role of SLs in plant development [11,12,20,24].

Furthermore, *zmccd8* always exhibited lower CHL content compared to WT, especially in younger leaves, and this behaviour was more pronounced in *zmccd8* plants subjected to N-deprivation. In fact, while WT displayed similar contents of CHL in young leaves in both

N-supplied and N-starved plants, *zmccd8* with N-deficiency displayed a clear drop in CHL levels with respect to *zmccd8* supplied with urea. Senescence, induced by abiotic stress such as nutrient and water deficiency, implies the mobilisation of resources from older organs to the most active metabolic sinks of younger leaves [29–31]. N is a key building block for chlorophyll and proteins crucial for photosynthesis [32]. To cope with N deficiency, plants move N stored in older leaves to younger leaves and reproductive organs, causing the characteristic symptom of N deficiency known as chlorosis [33]. Our results suggest that the senescence-mediated response to N deficiency is dependent on the ability to produce SL upon N-shortage.

Anthocyanins (ANTH), a class of water-soluble flavonoid pigments known for their photoprotective and pollinator-attracting functions, also play a crucial role in plant response to N deficiency [34]. These pigments are synthesised in the cytosol via the phenylpropanoid pathway and accumulate in the vacuoles of plant cells, imparting a red-purple colouration to leaves under nutritional stress. Studies have shown that anthocyanin accumulation is a common response in plants experiencing N deficiency, acting as a protective mechanism against various stress factors. Recent studies have highlighted these compounds not only as scavengers of free radicals under stress conditions but also as key modulators of signalling pathways and resource allocation [35]. This dual role underscores their significance in optimising the growth and adaptation of plants under nutritional stress. Low N stress initiates a complex series of molecular interactions that lead to the activation of anthocyanin accumulation. When N is limited, plants adjust their resource allocation to prioritise metabolic pathways that enhance nutrient efficiency, and this redistribution often coincides with the synthesis of anthocyanins that prevent an early senescence phenotype [36].

In our results, WT plants subjected to N-shortage did not evidence any increase of ANTH contents. On the contrary, *zmccd8* displayed a higher content of ANTH from the beginning, and increased accumulation in younger leaves in response to N-deprivation from 63 to 81 days.

Overall, these results indicate that after 81 days from initial fertilisation, WT plants responded to N-shortage by better mobilising their resources and did not need to increase ANTH production. Additionally, WT seems to also activate an additional cross-protection mechanism to preventively protect itself from water stress. These features were not observed in *zmccd8* plants, leading us to suppose the crucial involvement of SLs. Actually, there is a strong connection between the tolerance to N deficiency and water stress. For instance, plants may find it difficult to properly control stomata movement when the N supply is insufficient, leading to excessive water loss even under normal watering conditions [37]. Studies have shown that N deficiency can induce several defence mechanisms that enhance water stress tolerance. According to Xu et al. [38], N deficiency can lead to the increased production of abscisic acid (ABA), the key hormone in regulating stomatal closure and reducing water loss. Additionally, N deficiency has been associated with the accumulation of osmolytes such as proline, which help maintain cell turgor and protect cellular structures during water stress [39]. Finally, studies on gene expression have also revealed that N deficiency can trigger the upregulation of stress-responsive genes, including those involved in osmotic adjustment, cell wall remodelling, and protein stabilisation, which collectively enhance plant resilience to both N deficiency and drought [40].

In our results, *zmccd8* plants, which are impaired in their ability to efficiently mobilise N, exhibited signs of increased water stress, likely due to their inability to regulate stomatal closure effectively and prevent excessive water loss [16]. Moreover, our results suggest that the reduction of evapotranspiration and the lowering of stomatal conductance occurring in response to N deprivation in WT plants depend on SL production [41], since it did not occur in *zmccd8*.

Accordingly, foliar application of the synthetic SL GR24 helped to mitigate drought stress in various maize hybrids by improving chlorophyll content and gas exchange activity through the induction of stomatal closure during drought stress [41]. Moreover, elevated SL levels in shoots were predicted to enhance plant sensitivity to ABA, leading to a decrease in stomatal conductance and, consequently, improved plant survival [42].

To further confirm the involvement of SL in determining the response to the N absence observed in WT, the transcription of three genes involved in SL biosynthesis and signalling was assessed. The results showed that at 81 days from sowing, the expression of *CCD8* was always significantly higher in WT plants that were not supplied with urea compared to those fertilised, confirming that *CCD8* is a good marker for SL production [12,13]. A similar trend was also observed for *CCD7* and *MAX2* in both genotypes, while the transcription of *CCD8* was completely absent in *zmccd8*, as expected. The molecular data therefore confirm the above-hypothesised increase in SL metabolism after 81 days of growth in suboptimal N conditions.

Furthermore, the results showed a significantly lower transcription of *SULTR6* in N-deprived *zmccd8* plants after 81 days; *SULTR6* was both regulated by SLs [20,43] and induced upon water stress [44], and it might be implicated in the inability to activate the cross response to water stress observed for this mutant. Further evidence should be gained to consolidate this hypothesis.

4. Materials and Methods

4.1. Maize Growth Conditions

In this study, the maize inbred line B73 (*Zea mays* L.) and the *zmccd8::Ds* insertion mutant line in the B73 background were utilized. The *zmccd8::Ds* allele in B73 was created by backcrossing into B73 for 6 generations [24]. Then, 50 plants of the inbred line B73 (*Zea mays* L.) and 50 *zmccd8* mutant plants, herein referred to as wild-type (WT) and *zmccd8*, respectively, were grown in an open field during the spring-summer 2023 in Azienda Agraria Sperimentale L. Toniolo (45.3546335107061, 11.950633144227764, Legnaro, PD, Italy). The two genotypes were sown in April 2023 in a greenhouse, and, at about 15 days after sowing, they were moved to the open field. A week before transplanting from the greenhouse to the field, background fertilisation with an NPK mineral fertiliser of the 8-24-24 type was carried out on the soil. During the transplant, each plant was positioned 25 cm from each other in two rows spaced 1.5 m apart in order to separate the WT from the *zmccd8* mutant; then, within each row, each genotype was further divided in half to allow for a consistent fertilisation with urea (46% of N content, Cauvin Agricoltura, Genoa, Italy) of just the half plants for each genotype, thus creating two different levels of N provision in the soil. The fertilisation with urea as the N source for half of the plants took place after 58 days after sowing (DAS) from the greenhouse into the open field. Hence, four different conditions were obtained: WT +N and *zmccd8* +N ("+N" standing for N supply), WT -N and *zmccd8* -N ("-N" standing for N-deficiency). Drip lines and an anti-hail net were placed, and the plot was also fenced to protect the crop from wild animals.

4.2. Phenotypical Analysis

The WT and *zmccd8* height, internode length, stem circumference, and leaf number were assessed at 38, 45, 53, 60, 68, 81, and 89 days after sowing (DAS). The height was measured from the soil to the last fully developed node of the plants. The internode length was calculated by dividing the height by the number of the plant's leaves. The stem circumference was measured at the bottom of the plant, where it reached its maximum. For the leaf number, we excluded the cotyledon and the newest leaves that were not developed enough. At the end of the season, ears were collected and evaluated, and the kernels were weighted. Data were presented as the average of 25 independent biological replicates; each replicate considered a single plant as a biological replicate for each treatment (n = 25) $\pm$ standard error. For statistical analysis, data were considered significant when $p < 0.05$ using the ANOVA test performed with Fisher's least significant difference (LSD) multiple comparison method with R-Studio (R version 4.3.1 with the library (agricolae) [45].

4.3. Optic Measurements of Chlorophyll and Anthocyanins in Leaves

DUALEX SCIENTIFIC+^{TM} (Force-A, Orsay, France) was used to evaluate chlorophyll (CHL) and anthocyanin (ANTH) content at 38, 45, 53, 60, 68, 81, and 89 days after sowing (DAS).

The average of two readings was carried out in each leaf, starting from the basal part of the plant, so that leaf 1 was the closest to the ground, leaf 2 immediately above, and so on. Then, all data were grouped for statistical similarity among leaves in four homogeneous classes: L1-2, L3-4-5, L6-7-8, and L9-10-11. Data were presented as the average of 25 independent biological replicates, each replicate considered a single plant as a biological replicate for each treatment (n = 25) ± standard error. Statistical analysis was performed as previously described for the phenotypical analysis.

4.4. Stomatal Conductance, Transpiration Rate, and Photosystem II Efficiency Leaf Analysis

A LI-600 Porometer/Fluorometer (LI-COR Inc., Lincoln, Nebraska USA) was used to measure three different aspects of leaf photosynthesis, namely the stomatal conductance (*gsw*), the leaf transpiration rate (*E-app*), and the Photosystem II efficiency (*PhiPS II*), at 38, 45, 53, 60, 68, 81, and 89 days after sowing (DAS).

As for the optic measurements of chlorophyll and anthocyanins, two readings were carried out in each leaf starting from the basal part of the plant, so that leaf 1 was the closest to the ground, leaf 2 immediately above, and so on. Then, all data were grouped for statistical similarity among leaves in four homogeneous groups, namely leaves 1-2, leaves 3-4-5, leaves 6-7-8, and leaves 9-10-11.

Data were presented as the average of 25 independent biological replicates, each replicate considered a single plant as a biological replicate for each treatment (n = 25) ± standard error. Statistical analyses were performed as previously described for the phenotypical analysis.

4.5. RNA Extraction and cDNA Synthesis

For gene expression analysis, tissues of every leaf from three different plants for each condition of WT and *zmccd8* were sampled at 68, 81, and 89 DAS, which corresponded to 10, 23, and 32 days after urea supply (DAU), respectively. Each plant was considered a biological replicate. From the pool of leaves, 100 mg were sampled to extract the total RNA. A Spectrum™ Plant Total RNA Kit (Merck KGaA, Darmstadt, Germany and its subsidiary Sigma-Aldrich®) was used following the manufacturer's instructions. Total RNA was then quantified with a Nanodrop1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA) and evaluated qualitatively by agarose gel electrophoresis. Then, cDNA was synthesised from 500 ng of total RNA mixed with 1 µL of 10 µM oligo-dT, as described by Manoli et al. [46].

4.6. Gene Selection for Gene Expression Analysis

Three genes involved in SL biosynthesis (*CCD7*, *CCD8*, *MAX2*) and one gene positively related to drought stress (*SULTR6*) were selected according to their functions and/or to their transcriptional profiles in previous experiments (Table 1).

Table 1. List of maize genes and primers selected for expression analysis at the mRNA level.

Gene ID	Maize GDB ID	Functions	Primers	References
CCD7	Zm00001eb074640	Carotenoid cleavage dioxygenase 7, involved in SL biosynthesis	TCCGGCTCGCGCAGATTC CTGCCCAGAACCCATGGA	[12,13,43,47]
CCD8	Zm00001eb153000	Carotenoid cleavage dioxygenase 8, involved in SL biosynthesis	AGAAAGGTGTCTCTGCTGCT CTATGGGCTCGCTCACATGA	[12,13,24,43]
MAX2	Zm00001eb376660	Encoding F-box protein MAX2 involved in SL signaling	GAACAAGACCGGCATCCAAC TTAACTCGTCAGGCCTCCAG	[48]
SULTR6	Zm00001eb154590	Sulfate Transporter 6, mediates the uptake and translocation of sulfate	TAGGCGTCTTCAGGTTAGGG GAGGTCTGTCTTTGGCGTGA	[20,43,44,49]
MPE	Zm00001eb257640	Housekeeping gene, encoding the membrane protein PB1A10.07c	TGTACTCGGCAATGCTCTTG TTTGATGCTCCAGGCTTACC	[46]

4.7. Quantitative Reverse Transcription PCR (qRT-PCR)

Gene expression analyses were carried out at 68, 81, and 89 DAS, which corresponded to 10, 23, and 32 days after urea supply (DAU), respectively. qRT-PCR was performed using the StepOne Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) as described by Nonis et al. [50]. SYBR Green reagent (Applied Biosystems, Monza, Italy) was used according to the manufacturer's instructions. cDNA (2.5 ng) was used as a template and three technical repetitions were performed on three biological repetitions. The absence of multiple products and primer dimers was confirmed using melting curve analysis. Relative expression of the target gene was determined according to the Livak and Schmittgen [51] method, using *MEP* (*membrane protein PB1A10.07c*, Zm00001d018359) as a housekeeping gene, according to Manoli et al. [46]. Primers were designed using the Primer3 web tool (version 4.0.0; https://bioinfo.ut.ee/primer3/ accessed on 19 January 2024) [52]. For statistical analysis, data were considered significant when $p \leq 0.05$, using the Student's t test for pairwise comparisons. The genes analysed and the sequences of the relative primers used in qRT-PCR are reported in Table 1.

5. Conclusions

In conclusion, this research evidenced the prominent role of SLs in maize growth in fields but also highlighted their importance in regulating, at least in part, the response of this species to N availability. The production of SLs during N-starvation appears to be crucial for inducing senescence, allowing the reallocation of resources to younger tissues, and activating additional protective mechanisms against other stresses.

Future work is needed to explore the potential impact of these findings in agriculture, particularly in relation to improving crop resilience to nutrient limitations and abiotic stresses. Investigating the molecular pathways and genetic regulation linked to SLs could open new avenues for optimising plant performance under challenging growing conditions, such as drought or low-nitrogen soils. Additionally, examining the interaction between SLs and other phytohormones—such as abscisic acid, ethylene, and jasmonic acid—along with stress-responsive compounds such as anthocyanins could reveal synergistic effects that enhance stress tolerance. This knowledge could inform the development of crop varieties with improved efficiency in resource use, contributing to more sustainable and productive agricultural systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/stresses4040039/s1.

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