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Nano-Biochar Mediated Regulation of Antioxidant Defense System and Oxidative Stress Responses in Maize (*Zea mays* L.) Under Drought Stress Conditions

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Abstract | **Original Research Article**

Drought stress promotes the overproduction of reactive oxygen species (ROS), causing oxidative damage that limits maize growth and productivity. This study evaluated the effects of foliar-applied nano-biochar and sulphur on oxidative stress and antioxidant defense in maize (*Zea mays* L. cv. FH-1046) under drought. A pot experiment was conducted in a completely randomized design with two moisture regimes (control, 75–80% field capacity; drought, 35–40% field capacity) and three foliar treatments (distilled water, 3% nano-biochar, 12 mg L^{-1} sulphur), with five replicates. Foliar sprays were applied 50 days after emergence, and leaf biochemical traits were measured 15 days later. Drought increased hydrogen peroxide (H_2O_2) content by 24% on average ($P < 0.001$), but the increase was confined to sulphur-treated plants (765.9 vs. 491.3 nmol g^{-1} FW under well-watered conditions). Sulphur increased superoxide dismutase activity by 32% relative to the water spray, whereas nano-biochar increased peroxidase activity under drought by 83%. Drought induced a 4.6-fold increase in ascorbate peroxidase activity in unsprayed plants, but not in plants sprayed with nano-biochar or sulphur, and both sprays lowered total phenolics, mainly under well-watered conditions. Malondialdehyde, ascorbic acid, soluble sugars and free amino acids were not significantly affected. Neither spray reduced H_2O_2 or malondialdehyde, so these results do not demonstrate improved oxidative status or drought tolerance. The effects of foliar nano-biochar and sulphur on antioxidant responses were trait-specific and depended on water availability, and their consequences for growth and yield require further testing.

Keywords: Maize; drought stress; nano-biochar; sulphur; antioxidant enzymes; oxidative stress; hydrogen peroxide; phenolics.

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1. INTRODUCTION

Drought is among the most damaging abiotic stresses for crop production, impairing photosynthesis, nutrient uptake and growth (Farooq *et al*., 2009). Maize (*Zea mays* L.) is particularly important and exposed. Together with wheat, it accounted for more than half of global cereal production in 2013, with maize alone at roughly 1,016 million tons (Daryanto *et al*., 2016). A global synthesis of field experiments found that maize lost about 39% of its yield under a water reduction of roughly 40%, a considerably larger loss than in wheat (about 21%) (Daryanto *et al*., 2016). In maize hybrids, drought has also been shown to cause oxidative stress through excessive ROS production, which reduces growth and yield formation (Anjum *et al*., 2017). Sustaining maize performance under water-limited conditions therefore requires practical strategies that protect plant physiological function.

At the cellular level, drought disturbs the balance between the production and scavenging of reactive oxygen species (ROS). Water deficit disrupts photosynthesis and increases photorespiration, which raises ROS production in chloroplasts, mitochondria and peroxisomes (Apel & Hirt, 2004; Miller *et al*., 2010). The ROS involved include superoxide radicals, hydrogen peroxide (H_2O_2), hydroxyl radicals and singlet oxygen (Gill & Tuteja, 2010; Mittler, 2002). At low

concentrations ROS act as signals, but when they exceed the capacity of antioxidant systems they damage lipids, proteins and nucleic acids (Apel & Hirt, 2004; Mittler, 2002). H_2O_2 is widely used as an indicator of oxidative stress because it is relatively stable and can diffuse across compartments (Smirnoff & Arnaud, 2019). In maize, prolonged drought leads to ROS accumulation, oxidation of the glutathione pool and a decline in leaf glutathione (Ahmad *et al*., 2016). At the same time, the relationship between drought and oxidative stress is not simple, because the roles of different ROS-generating processes and the specificity of antioxidant networks remain incompletely understood (Noctor *et al*., 2014).

Plants counter oxidative stress with enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD) converts superoxide into H_2O_2 (Alscher *et al*., 2002), which is then detoxified by catalase (CAT), ascorbate peroxidase (APX) and peroxidases (POD) (Gill & Tuteja, 2010). APX uses ascorbate as its electron donor and is a central component of the ascorbate–glutathione cycle (Caverzan *et al*., 2012), whereas peroxidases form a large family with functions beyond H_2O_2 scavenging (Passardi *et al*., 2005). Among non-enzymatic antioxidants, ascorbic acid and glutathione act as redox buffers (Foyer & Noctor, 2005, 2011), and phenolic compounds accumulate under abiotic stress and scavenge ROS (Sharma *et al*., 2019). Oxidative damage to membranes is commonly assessed through malondialdehyde (MDA) content, a widely used lipid peroxidation marker (Morales & Munné-Bosch, 2019), and through membrane permeability measured as electrolyte leakage (Bajji *et al*., 2002). Because the responses of individual enzymes and metabolites differ, assessing several enzymatic and non-enzymatic components together is more informative than relying on a single marker.

Biochar, a carbon-rich product of biomass pyrolysis, improves soil physical properties, water retention and nutrient availability (Lehmann & Joseph, 2015). Reviews report that biochar improves abiotic stress tolerance partly by modulating ionic homeostasis, the photosynthetic apparatus and antioxidant machinery, and by reducing oxidative damage (Imran *et al*., 2023). In maize, biochar amendment has improved yield through better plant–soil moisture relations (Haider *et al*., 2015). In a field study in Pakistan, activated biochar applied to soil under reduced irrigation was associated in maize with higher membrane stability, lower MDA, higher SOD, POD and CAT activities, and about 26% higher grain yield than unamended soil; the 5 t ha^{-1} dose outperformed 10 t ha^{-1} (Naeem *et al*., 2024). These results show that benefits depend on dose and that antioxidant responses are part of how biochar supports maize under water deficit.

Reducing biochar to the nanoscale has attracted growing interest. Nano-biochar has been described as having greater surface area, adsorption capacity and mobility in soil than traditional fertilizers, although its risks and potential toxicity also need evaluation (Faizan *et al*., 2024). In drought-stressed rice, combining soil-applied nano-biochar with foliar calcium oxide nanoparticles reduced MDA by 56% and H_2O_2 by 59% (Bilge *et al*., 2026). However, this was a combined treatment, and in the studies cited above biochar was applied to the soil. The effect of nano-biochar applied directly to leaves of maize under drought has received comparatively little attention.

Sulphur is an essential nutrient and a constituent of cysteine and methionine, from which glutathione is synthesised. Sulphur-containing defence compounds, including glutathione, phytochelatins and various secondary metabolites, are central to stress defence, and their formation depends on sulphur supply and assimilation (Capaldi *et al*., 2015). Sulphur and its derivatives have been reported to improve antioxidant defence against excess ROS under several abiotic stresses (Hasanuzzaman *et al*., 2018). For example, excess sulphur supply improved photosynthesis and growth in salt-stressed mustard through increased glutathione production (Fatma *et al*., 2014), and sulphur supply influenced antioxidant activity in cadmium-stressed Indian mustard (Bashir *et al*., 2015). Sulphur assimilation is also closely tied to drought responses (Chan *et al*., 2013), and in maize drought causes differential acclimation of sulphur and glutathione metabolism in leaves and roots (Ahmad *et al*., 2016). Supplying sulphur may therefore support redox buffering under drought, which makes foliar sulphur a useful comparison treatment for nano-biochar.

To our knowledge, the effects of foliar nano-biochar and foliar sulphur on oxidative stress and antioxidant defence in maize under drought have not been directly compared. Therefore, this study evaluated the effects of foliar nano-biochar (3%) and sulphur (12 mg L^{-1}) on oxidative stress markers (H_2O_2, MDA and membrane permeability), enzymatic antioxidants (SOD, APX and POD) and non-enzymatic antioxidants (phenolics and ascorbic acid) in maize grown under well-watered (75–80% field capacity) and drought (35–40% field capacity) conditions. We hypothesised that foliar nano-biochar would strengthen antioxidant defence and limit oxidative stress under drought more than sulphur.

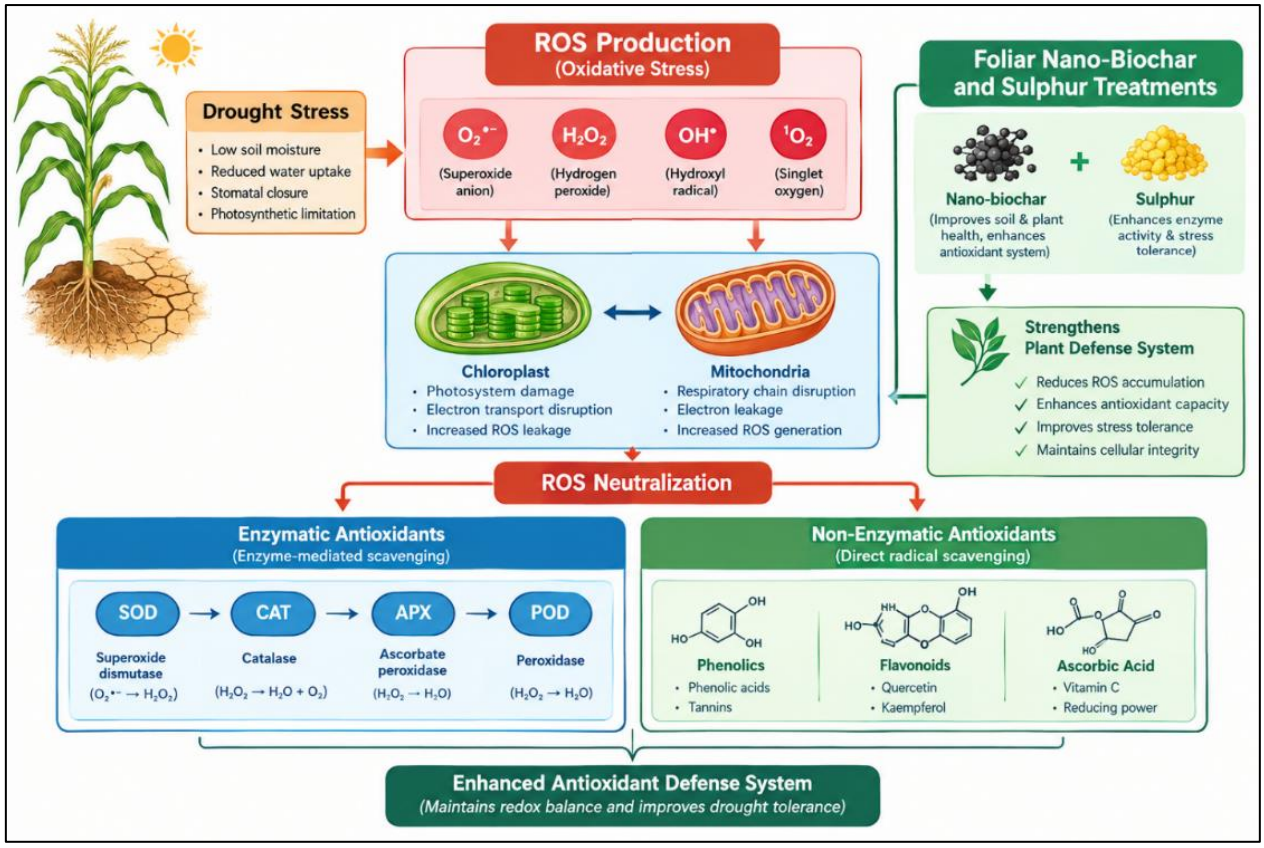


Figure 1: Conceptual pathway of drought-induced ROS generation and enzymatic/non-enzymatic antioxidant defense in maize, highlighting nano-biochar and sulphur intervention points

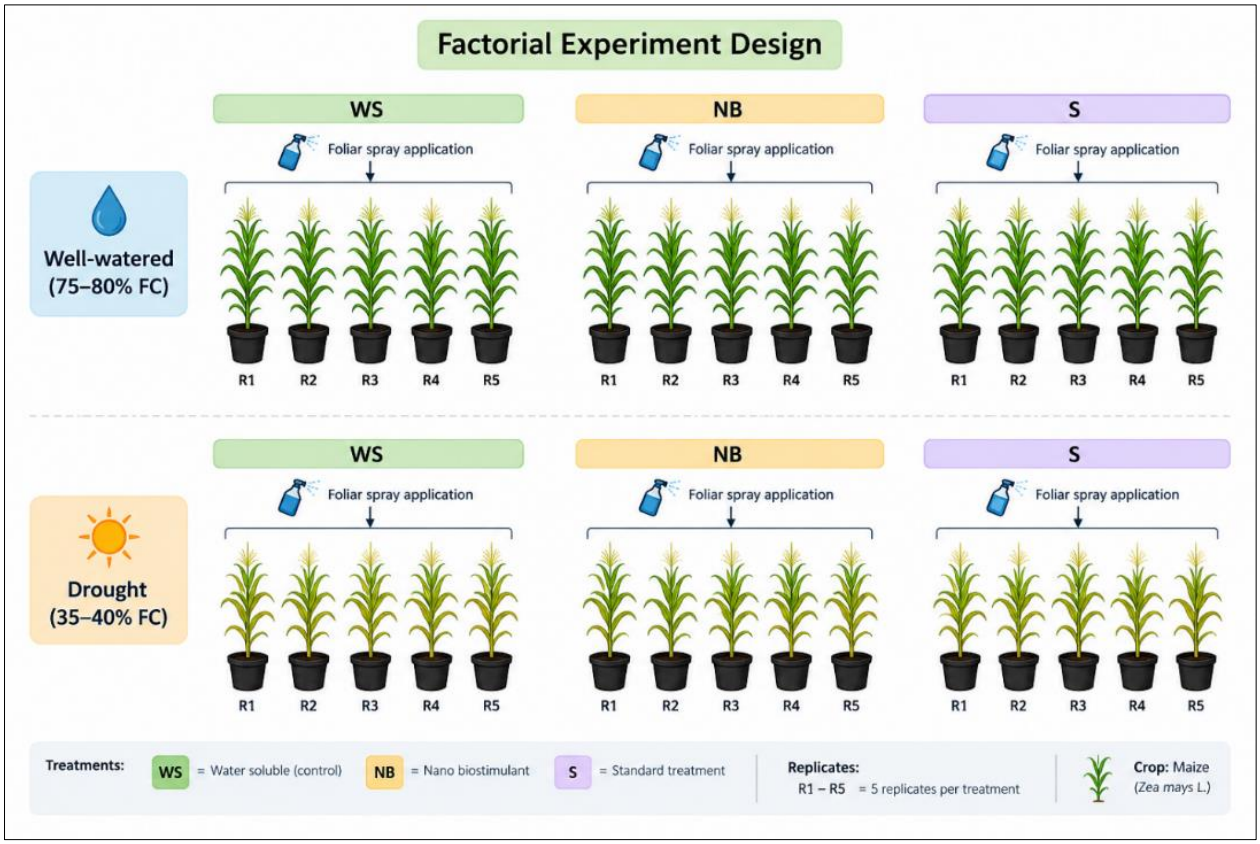


Figure 2: Schematic of the factorial pot experiment: two water regimes (well-watered vs drought) × three foliar treatments (WS, NB, S) with five replicates

2. MATERIALS AND METHODS

2.1 Experimental site and plant material

A pot experiment was conducted at Government College University, Faisalabad, Pakistan, to determine how foliar sulphur and nano-biochar modulate the biochemical responses of maize (Zea mays L.) under limited water supply. Each pot was filled with 10 kg of sandy loam soil. Seeds of maize cultivar FH-1046 were obtained from the Ayub Agricultural Research Institute (AARI), Faisalabad. Ten seeds were sown per pot at a spacing of 3 inches (≈7.6 cm), and the pots were lightly sprinkled with water after sowing. After germination and initial establishment, seedlings were thinned to six healthy, uniform plants per pot.

2.2 Experimental design and water regimes

The experiment was laid out in a completely randomised design with a two-factor factorial arrangement: two water regimes × three foliar treatments, with five replicates per treatment combination (30 pots in total). Soil moisture was maintained at 75–80% field capacity (FC) in the well-watered control and at 35–40% FC in the drought treatment.

2.3 Foliar treatments

Three foliar treatments were applied: distilled water (water spray, WS; control), 3% nano-biochar (NB) and 12 mg L^{-1} sulphur (S). Nano-biochar is generally produced by pyrolysis of biomass followed by mechanical size reduction of the parent biochar, most commonly by ball milling, and its properties depend on the pyrolysis conditions (Kumar *et al*., 2020; Ramanayaka *et al*., 2020). Foliar sprays were applied 50 days after emergence.

2.4 Sampling

Leaf samples for biochemical analyses were collected 15 days after foliar application. Each analysis was performed on the five replicates of each treatment combination.

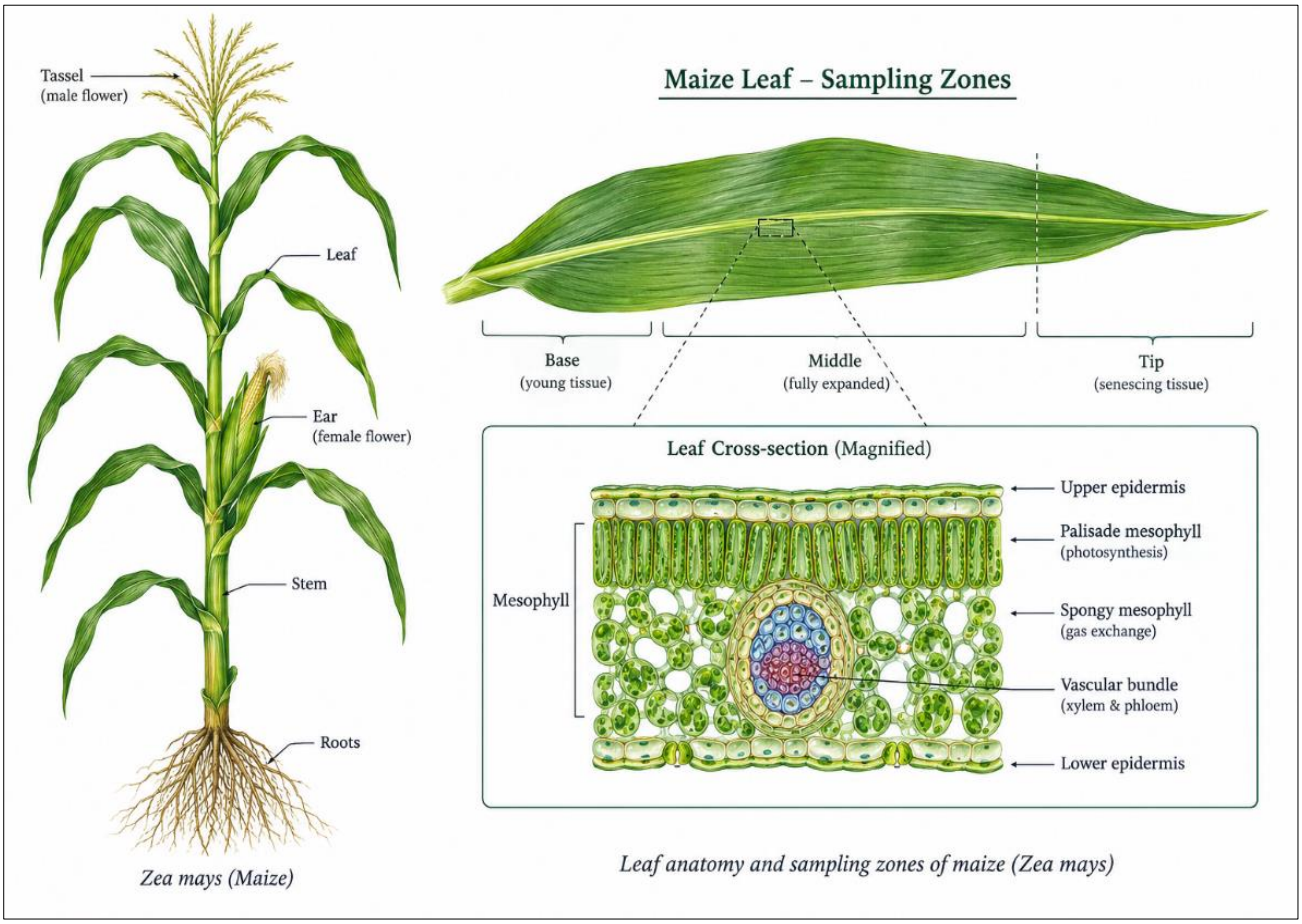


Figure 3: Maize leaf sampling diagram showing fully expanded young leaves collected 15 days after foliar application for biochemical analysis

2.5 Oxidative stress markers

2.5.1 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) content was determined following Velikova *et al*. (2000). Fresh leaf tissue (0.5 g) was homogenised in 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) in an ice bath, and the homogenate was centrifuged at 12,000 rpm for 15 min. The reaction mixture contained 0.5 mL of supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). Absorbance was

read at 390 nm using a spectrophotometer (U-2001, Hitachi), and H_2O_2 content was calculated from a standard curve prepared with known H_2O_2 concentrations.

2.5.2 Malondialdehyde

Malondialdehyde (MDA) was determined according to Dhindsa *et al*. (1981) with minor modifications and with the correction for interfering compounds of Hodges *et al*. (1999). Leaf tissue (0.5 g) was homogenised in 6 mL of 10% TCA and centrifuged at 4000 rpm for 20 min. A 2 mL aliquot of the supernatant was mixed with 2 mL of 0.6% thiobarbituric acid (TBA), heated at 100 °C for 20 min, and rapidly cooled on ice for 10–20 min. The mixture was centrifuged again at 10,000 rpm for 10 min, and the absorbance of the supernatant was read at 532, 600 and 450 nm. MDA concentration was calculated as 6.45 × (A532 − A600) − 0.56 × A450 and expressed as µmol g^{-1} fresh weight (FW).

2.5.3 Relative membrane permeability

Relative membrane permeability (RMP) was determined using an electrolyte-leakage approach (Bajji *et al*., 2002) from 0.5 g of leaf tissue cut into small pieces and placed in 10 mL of distilled water. After vortexing, the initial electrical conductivity (EC_0) was measured. The tubes were then kept at 4 °C overnight, and EC_1 was recorded. The samples were subsequently autoclaved for 20 min, cooled, and EC_2 was measured. RMP was calculated as:

$$RMP\ (\%) = [(EC_1 - EC_0) / (EC_2 - EC_0)] \times 100$$

2.6 Enzymatic antioxidants

For enzyme assays, 0.5 g of fresh leaf tissue was ground with a mortar and pestle in 5 mL of cold 50 mM phosphate buffer (pH 7.8) containing 2% (w/v) polyvinylpolypyrrolidone (PVP). The homogenate was centrifuged at 13,000 × g for 20 min at 4 °C, and the supernatant was used as the enzyme extract. Where activities are expressed per mg protein, protein content of the extract was determined by the method of Bradford (1976).

2.6.1 Superoxide dismutase

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed following Giannopolitis and Ries (1977). The 3 mL reaction mixture contained 13 µM methionine, 63 µM nitroblue tetrazolium chloride (NBT), 1.3 µM riboflavin, 50 mM phosphate buffer (pH 7.8) and enzyme extract. The mixture was illuminated for 10 min under fluorescent light (80 µmol m^{-2} s^{-1}), and absorbance was read at 560 nm (UV-2010, Hitachi, Japan). One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the photochemical reduction of NBT. Activity was expressed per g FW.

2.6.2 Peroxidase

Peroxidase (POD, EC 1.11.1.7) activity was measured according to Chance and Maehly (1955). The reaction mixture consisted of 1.9 mL of phosphate buffer, 100 µL of guaiacol, 100 µL of H_2O_2 and 100 µL of enzyme extract. The change in absorbance at 470 nm was recorded for 120 s using a spectrophotometer, and activity was expressed as U mg^{-1} protein.

2.6.3 Ascorbate peroxidase

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined following Asada and Takahashi (1987). The 1 mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 200 µL of enzyme extract. The decrease in absorbance at 290 nm, corrected for the small non-enzymatic oxidation of ascorbate by H_2O_2, was monitored against a blank (extinction coefficient 2.9 mM^{-1} cm^{-1}). Activity was expressed as U mg^{-1} protein, where one unit corresponds to a change in absorbance of 0.1 min^{-1}.

2.7 non-enzymatic antioxidants and osmolytes

2.7.1 Ascorbic acid

Ascorbic acid (AsA) was determined according to Mukherjee and Choudhuri (1983) with slight modifications. Leaf tissue (0.5 g) was homogenised in 7 mL of 10% TCA and centrifuged at 4000 rpm for 20 min. A 3.5 mL aliquot of the supernatant was mixed with 0.5 mL of DTC reagent (2 g 2,4-dinitrophenylhydrazine, 4 g thiourea and 0.08 g copper sulphate per 100 mL of 9 N sulphuric acid) and incubated at 37 °C for 3 h. After rapid cooling on ice for 10 min, 2.5 mL of ice-cold 65% sulphuric acid was added drop by drop with thorough mixing, and the mixture was held at 30 °C for 30 min. After centrifugation at 15,000 rpm for 20 min, the colour was read at 520 nm. AsA was expressed as µg g^{-1} FW.

2.7.2 Total phenolics

Total phenolic content was determined with the Folin–Ciocalteu reagent (FCR) following Slinkard and Singleton (1977). A 1 mL aliquot of methanolic extract (containing 1 mg of extract) was mixed with 46 mL of distilled water and 1 mL of FCR. After 3 min, 3 mL of 2% sodium carbonate was added, and the mixture was shaken intermittently for 2 h at room temperature. Absorbance was read at 760 nm, and phenolics were calculated from a standard curve and expressed as pyrocatechol equivalents per g FW.

2.7.3 Total soluble sugars

Total soluble sugars were estimated by the anthrone method of Yoshida *et al*. (1976). The anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 mL of 95% sulphuric acid. Four millilitres of freshly prepared reagent were added to 1 mL of extract, and the tubes were stoppered, vortexed, heated for 10 min in a boiling water bath, and cooled rapidly. Absorbance was read at 630 nm, and sugar content was

calculated from a glucose standard curve (10–100 ppm) prepared from a stock glucose solution.

2.7.4 Total free amino acids

Total free amino acids were determined by the ninhydrin method (Lee & Takahashi, 1966). An aliquot of leaf extract was reacted with ninhydrin reagent in a boiling water bath, the mixture was cooled, and absorbance was read at 570 nm. Amino acid content was calculated from a standard curve (glycine) and expressed as mg g^{-1} FW.

2.8 Statistical analysis

Data were analysed by two-way analysis of variance (ANOVA) appropriate to a factorial completely randomised design, with water regime and foliar application as factors, using CoStat software. Where the ANOVA indicated significant effects, treatment means were compared using the least significant difference (LSD) test at $P \leq 0.05$. Data are presented as means ± standard error (n = 5).

3. RESULTS

3.1 Overview of treatment effects

The effects of water regime (D), foliar application (FA) and their interaction (D × FA) on the ten biochemical traits are summarised in Table 1, and treatment means are given in Table 2 and Figures 4-6. Drought, foliar application and their interaction significantly affected membrane permeability and ascorbate peroxidase activity. H_2O_2 was affected by water regime and by the D × FA interaction; SOD by foliar application alone; and POD and total phenolics by the interaction (phenolics also by foliar application). MDA, ascorbic acid, total soluble sugars and total free amino acids were not significantly affected by any factor.

Table 1: Summary of ANOVA (F values) for the effects of water regime (D), foliar application (FA) and their interaction on oxidative stress markers and antioxidant traits of maize. Error df = 24 for all traits

Trait	Water regime (D) (df = 1)	Foliar application (FA) (df = 2)	D × FA (df = 2)
Hydrogen peroxide (H_2O_2)	14.19***	1.08 ns	4.94*
Malondialdehyde (MDA)	0.00 ns	0.28 ns	0.31 ns
Membrane permeability (RMP)	521.98***	12690.24***	408.68***
Superoxide dismutase (SOD)	1.99 ns	4.10*	1.03 ns
Peroxidase (POD)	1.49 ns	2.19 ns	4.31*
Ascorbate peroxidase (APX)	4.28*	12.25***	27.94***
Total phenolics	3.53 ns	46.43***	6.31**
Ascorbic acid (AsA)	0.94 ns	1.23 ns	0.69 ns
Total soluble sugars	0.48 ns	0.60 ns	1.94 ns
Total free amino acids	1.78 ns	0.69 ns	0.90 ns

*ns, not significant; * P < 0.05; ** P < 0.01; *** P < 0.001.*

3.2 Oxidative stress markers

Drought significantly increased H_2O_2 content ($F = 14.19$, $P < 0.001$), whereas foliar application had no overall effect ($P = 0.354$); the significant D × FA interaction ($P < 0.05$) showed that the response to the foliar sprays depended on water availability (Table 1; Figure 4a). Averaged over foliar treatments, H_2O_2 was 24% higher under drought than under well-watered conditions (660 vs. 531 nmol g^{-1} FW). This increase was driven by sulphur-treated plants: under drought, S plants accumulated the most H_2O_2 (765.9 nmol g^{-1} FW), 28% more than WS plants (599.3) and 56% more than well-watered S plants (491.3). NB plants under drought (614.5) did not differ from WS plants, and WS plants themselves did not differ between water regimes (599.3 vs. 581.1). Under well-watered conditions, H_2O_2 did not differ significantly among foliar treatments (Table 2).

MDA content was not significantly affected by water regime ($F = 0.00$, $P = 0.968$), foliar application ($F = 0.28$, $P = 0.761$) or their interaction ($F = 0.31$, $P = 0.735$), and means remained between 0.62 and 0.76 µmol g^{-1} FW across all treatments (Figure 4b).

Relative membrane permeability was significantly affected by water regime, foliar application and their interaction (all $P < 0.001$; Table 1; Figure 4c). The foliar treatment had by far the largest effect: S-treated plants showed values of 96.8–99.8% in both water regimes, compared with 39.6–40.7% in WS plants. NB did not change RMP relative to WS under drought (41.4% vs. 40.7%), but increased it under well-watered conditions (61.2% vs. 39.6%). WS plants did not differ between water regimes, so the lower mean RMP under drought (59.6% vs. 66.9% when averaged over sprays) reflected the lower values of NB and S plants under drought than under well-watered conditions.

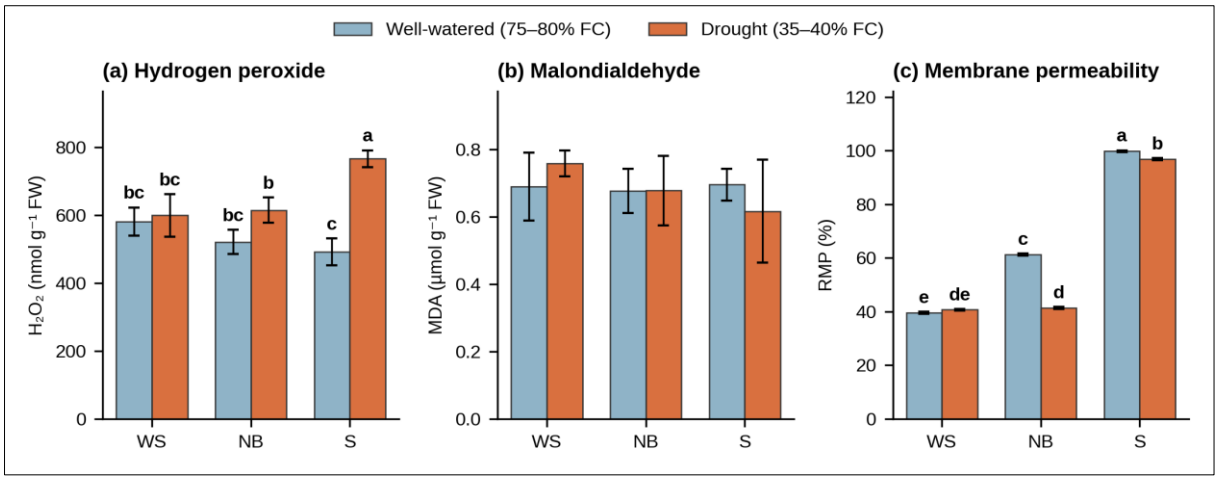


Figure 4: Effects of foliar nano-biochar (NB, 3%) and sulphur (S, 12 mg L⁻¹), compared with a water spray (WS), on (a) hydrogen peroxide, (b) malondialdehyde and (c) relative membrane permeability (RMP) in maize leaves under well-watered (75–80% FC) and drought (35–40% FC) conditions. Bars are means ± SE (n = 5). Different letters indicate significant differences among treatment combinations (LSD, P ≤ 0.05); panels without letters showed no significant effects

3.3 Enzymatic antioxidants

SOD activity was significantly affected by foliar application ($F = 4.10$, $P < 0.05$) but not by water regime ($P = 0.171$) or the interaction ($P = 0.374$) (Table 1; Figure 2a). Averaged over water regimes, S increased SOD activity by 32% relative to WS (4.08 vs. 3.10 U g⁻¹ FW; LSD for foliar means = 0.72), while NB (3.45 U g⁻¹ FW) was intermediate and did not differ significantly from either.

POD activity was not significantly affected by water regime ($P = 0.233$) or foliar application ($P = 0.133$), but the D × FA interaction was significant ($F = 4.31$, $P < 0.05$; Figure 2b). Under drought, NB plants showed the highest POD activity (56.1 U mg⁻¹ protein), 83% higher than WS plants (30.7), while S plants (50.8) did not differ significantly from either. Under well-watered conditions, POD did not differ significantly among foliar treatments, but NB plants had the lowest activity (22.8 U mg⁻¹ protein); their activity was 2.5-fold higher under drought than under well-watered conditions.

APX activity was significantly affected by water regime ($F = 4.28$, $P < 0.05$), foliar application ($F = 12.25$, $P < 0.001$) and their interaction ($F = 27.94$, $P < 0.001$; Figure 2c). Drought induced a marked increase in APX only in unsprayed plants (18.8 vs. 4.1 U mg⁻¹ protein in WS plants under well-watered conditions, a 4.6-fold increase). In drought-stressed plants sprayed with NB or S, APX remained low (3.6 and 3.9 U mg⁻¹ protein) and comparable to well-watered WS plants. Under well-watered conditions, S raised APX activity 2.4-fold relative to WS (9.7 vs. 4.1), whereas NB had no significant effect (5.1).

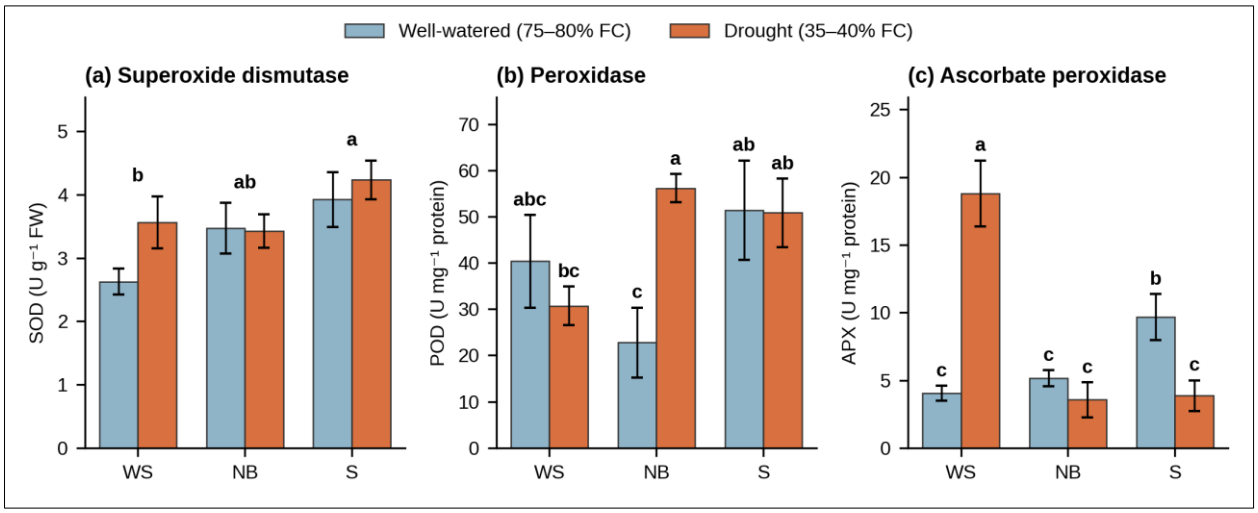


Figure 5: Effects of foliar nano-biochar (NB, 3%) and sulphur (S, 12 mg L⁻¹), compared with a water spray (WS), on the activities of (a) superoxide dismutase (SOD), (b) peroxidase (POD) and (c) ascorbate peroxidase (APX) in maize leaves under well-watered and drought conditions. Bars are means ± SE (n = 5). In (a), letters refer to foliar-treatment means averaged over water regimes; in (b) and (c), different letters indicate significant differences among treatment combinations (LSD, P ≤ 0.05)

3.4 non-enzymatic antioxidants and osmolytes

Total phenolic content was significantly affected by foliar application ($F = 46.43$, $P < 0.001$) and by the D × FA interaction ($F = 6.31$, $P < 0.01$), but not by water regime ($P = 0.072$) (Figure 3a). Unsprayed plants had the highest phenolic contents (11.0 mg g^{-1} FW under drought and 12.6 under well-watered conditions). Both foliar treatments lowered phenolics relative to WS, but the size of the reduction depended on water availability: S decreased phenolics by 49% under drought (5.6 mg g^{-1} FW) and by 67% under well-watered conditions (4.1), and NB decreased them by 55% under well-watered conditions (5.6), whereas under drought NB (9.1) did not differ significantly from WS.

Ascorbic acid ($F = 0.94$, 1.23 and 0.69 for D, FA and D × FA; all $P > 0.30$), total soluble sugars ($F = 0.48$, 0.60 and 1.94; all $P > 0.16$) and total free amino acids ($F = 1.78$, 0.69 and 0.90; all $P > 0.19$) were not significantly affected by water regime, foliar application or their interaction (Table 1; Figures 3b–d). Mean values ranged from 256 to 278 µg g^{-1} FW for ascorbic acid, from 11.9 to 12.6 mg g^{-1} FW for soluble sugars, and from 1.84 to 2.28 mg g^{-1} FW for free amino acids.

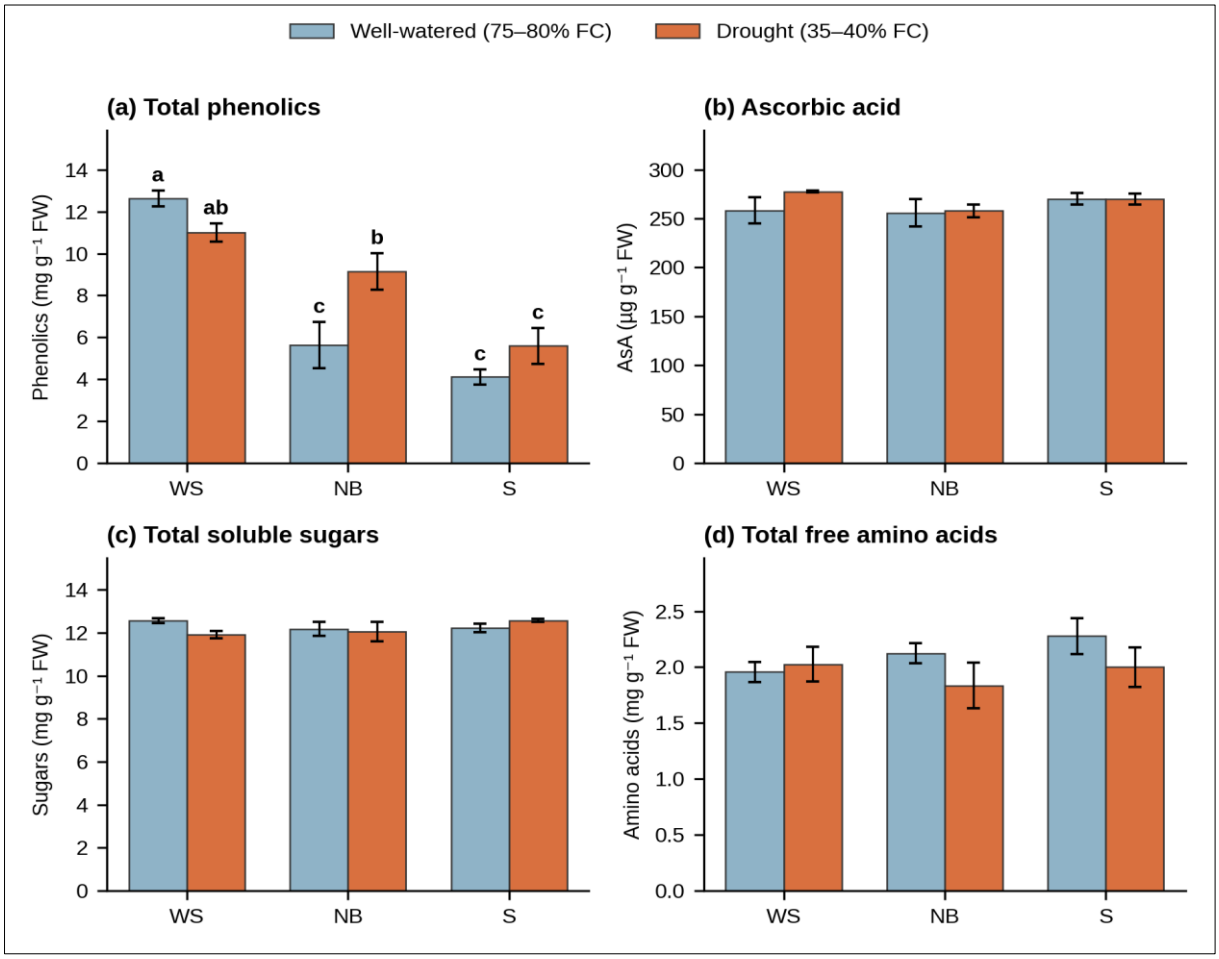


Figure 6: Effects of foliar nano-biochar (NB, 3%) and sulphur (S, 12 mg L^{-1}), compared with a water spray (WS), on (a) total phenolics, (b) ascorbic acid, (c) total soluble sugars and (d) total free amino acids in maize leaves under well-watered and drought conditions. Bars are means ± SE (n = 5). Different letters indicate significant differences among treatment combinations (LSD, $P \leq 0.05$); panels without letters showed no significant effects

Table 2: Means ± SE (n = 5) of oxidative stress markers and antioxidant traits of maize leaves under two water regimes and three foliar treatments (WS, water spray; NB, 3% nano-biochar; S, 12 mg L^{-1} sulphur). Different letters within a row indicate significant differences among treatment combinations (LSD, $P \leq 0.05$); letters are given only for traits with a significant D × FA interaction

Trait	Well-watered (75–80% FC)			Drought (35–40% FC)			LSD (5%)
	WS	NB	S	WS	NB	S	
H_2O_2 (nmol g^{-1} FW)	581.1 ± 41.5 bc	520.6 ± 36.0 bc	491.3 ± 39.4 c	599.3 ± 62.5 bc	614.5 ± 37.5 b	765.9 ± 24.9 a	122.3
MDA (µmol g^{-1} FW)	0.690 ± 0.101	0.677 ± 0.066	0.696 ± 0.047	0.759 ± 0.039	0.678 ± 0.103	0.617 ± 0.153	–

RMP (%)	39.6 ± 0.4 e	61.2 ± 0.4 c	99.8 ± 0.4 a	40.7 ± 0.3 de	41.4 ± 0.4 d	96.8 ± 0.4 b	1.1
SOD (U g^{-1} FW)	2.63 ± 0.21	3.47 ± 0.40	3.92 ± 0.43	3.56 ± 0.41	3.42 ± 0.27	4.24 ± 0.31	–
POD (U mg^{-1} protein)	40.3 ± 10.1 abc	22.8 ± 7.6 c	51.4 ± 10.8 ab	30.7 ± 4.2 bc	56.1 ± 3.0 a	50.8 ± 7.4 ab	22.5
APX (U mg^{-1} protein)	4.05 ± 0.56 c	5.14 ± 0.60 c	9.67 ± 1.72 b	18.80 ± 2.45 a	3.56 ± 1.30 c	3.87 ± 1.13 c	4.24
Total phenolics (mg g^{-1} FW)	12.63 ± 0.38 a	5.62 ± 1.11 c	4.12 ± 0.36 c	10.99 ± 0.44 ab	9.14 ± 0.88 b	5.60 ± 0.85 c	2.13
AsA (µg g^{-1} FW)	258.4 ± 13.4	255.8 ± 13.9	270.0 ± 5.9	277.6 ± 1.1	257.9 ± 6.5	270.0 ± 5.6	–
Total soluble sugars (mg g^{-1} FW)	12.57 ± 0.11	12.18 ± 0.33	12.23 ± 0.20	11.90 ± 0.17	12.06 ± 0.45	12.57 ± 0.08	–
Total free amino acids (mg g^{-1} FW)	1.96 ± 0.09	2.12 ± 0.09	2.28 ± 0.16	2.03 ± 0.15	1.84 ± 0.20	2.00 ± 0.18	–

LSD values apply to comparisons among the six treatment combinations; – indicates no significant interaction. For SOD, foliar-treatment means averaged over water regimes were 3.10 (WS), 3.45 (NB) and 4.08 (S) U g^{-1} FW (LSD = 0.72).

4. DISCUSSION

Drought disturbs the balance between the production and scavenging of reactive oxygen species (ROS), and plants respond through enzymatic and non-enzymatic antioxidants (Gill & Tuteja, 2010; Mittler, 2002). In this study, drought raised hydrogen peroxide (H_2O_2) in maize leaves, and the foliar sprays modified several antioxidant traits. However, the effects of nano-biochar and sulphur were trait-specific and depended on water availability, and neither spray lowered H_2O_2 or malondialdehyde (MDA). The hypothesis that foliar nano-biochar would limit oxidative stress under drought more effectively than sulphur was therefore not supported by these markers.

4.1 Oxidative stress markers

Averaged over foliar treatments, H_2O_2 was 24% higher under drought, in line with the ROS accumulation reported for drought-stressed maize (Ahmad *et al*., 2016; Anjum *et al*., 2017) and with the general view that drought disturbs ROS homeostasis (Miller *et al*., 2010; Mittler, 2002). The significant drought × foliar interaction, however, shows that this increase was not uniform. Unsprayed plants did not differ between water regimes, and the rise was confined to sulphur-treated plants, which had the highest H_2O_2 under drought. Because SOD converts superoxide into H_2O_2 (Alscher *et al*., 2002), the higher SOD activity of sulphur-treated plants may have contributed to this accumulation, although the present data cannot establish a causal link. Nano-biochar did not change H_2O_2 relative to the water spray under either water regime.

MDA was unaffected by drought, foliar treatment or their interaction. Higher H_2O_2 under drought was therefore not accompanied by detectable lipid peroxidation, which suggests that the stress was moderate or that the antioxidant system was sufficient to limit membrane lipid damage at the time of sampling. This is consistent with the view that the relationship between drought and oxidative damage is not straightforward (Noctor *et al*., 2014). It differs, however, from the oxidative stress reported for maize hybrids under drought maintained down to 40% of field capacity (Anjum *et al*., 2017), from the lower MDA reported for biochar-amended maize under reduced irrigation (Naeem *et al*., 2024), and from the reductions in MDA and H_2O_2 reported for drought-stressed rice treated with soil-applied nano-biochar combined with foliar calcium oxide nanoparticles (Bilge *et al*., 2026). Those studies differ from ours in cultivar, species, application route, treatment combination, stress duration and severity, so direct comparison should be made with caution. In addition, thiobarbituric acid-based MDA assays can be affected by interfering compounds (Hodges *et al*., 1999; Morales & Munné-Bosch, 2019), which is a general limitation of the method even though a correction was applied here.

Relative membrane permeability, an electrolyte-leakage measure commonly used to assess membrane stability under water stress (Bajji *et al*., 2002), was strongly affected by the foliar treatments. It was highest in sulphur-treated plants (about 97–100% in both regimes) and lower in unsprayed plants (about 40%), and nano-biochar increased it only under well-watered conditions. Unsprayed plants did not differ between water regimes. Since MDA did not change, these differences do not appear to reflect lipid peroxidation. They may reflect direct effects of the sprayed materials on the leaf tissue or on conductivity measurements rather than drought-induced membrane injury, and permeability should therefore be interpreted cautiously as a stress indicator in this experiment.

4.2 Enzymatic antioxidants

SOD, the first enzyme in the detoxification pathway (Alscher *et al*., 2002), responded to foliar application but not to drought. Sulphur increased SOD activity by about 32% relative to the water spray, whereas nano-biochar produced an intermediate, non-significant increase (about 11%). Sulphur supply has been associated with higher antioxidant activity and SOD expression in cadmium-stressed mustard (Bashir *et al*., 2015), which is consistent with the direction of the sulphur response here. A higher SOD activity under biochar has been reported in maize under reduced irrigation (Naeem *et al*., 2024), and the direction of the

nano-biochar response here is similar, but it was not statistically significant.

POD showed a clear dependence on water availability. Under drought, nano-biochar-treated plants had the highest POD activity, 83% above the water spray, whereas under well-watered conditions they had the lowest values. Peroxidases act downstream of SOD in H_2O_2 detoxification (Gill & Tuteja, 2010), but they also have other functions, including roles in cell wall metabolism and ROS production (Passardi *et al*., 2005), so changes in POD activity cannot be interpreted solely as H_2O_2 scavenging. Increased POD activity under biochar has also been reported in maize under water deficit (Naeem *et al*., 2024). The reversal between regimes suggests that nano-biochar effects on POD are context dependent and appear mainly when water is limiting.

The APX response was the most striking. Drought induced a 4.6-fold increase in APX in unsprayed plants, a typical activation of H_2O_2 scavenging under stress (Caverzan *et al*., 2012), but this induction was absent in plants sprayed with nano-biochar or sulphur. Lower H_2O_2 does not explain this, because sprayed plants did not have lower H_2O_2 than unsprayed plants. One possibility is that the sprays altered how H_2O_2 scavenging was regulated, for example by shifting detoxification towards other peroxidases, as the high POD activity of nano-biochar plants under drought might suggest. Under well-watered conditions, sulphur raised APX 2.4-fold. Sulphur is required for cysteine and glutathione synthesis (Capaldi *et al*., 2015), APX operates within the ascorbate–glutathione cycle (Caverzan *et al*., 2012; Foyer & Noctor, 2011), sulphur assimilation is closely linked to drought responses (Chan *et al*., 2013), and glutathione metabolism is part of the redox buffering network in maize leaves (Ahmad *et al*., 2016). These links could be relevant here, although glutathione was not measured. These interpretations remain hypotheses.

4.3 non-enzymatic antioxidants and osmolytes

Total phenolics were highest in unsprayed plants, and both sprays reduced them, most strongly under well-watered conditions. Phenolic compounds typically accumulate under abiotic stress and scavenge ROS (Sharma *et al*., 2019), so this result is the opposite of a simple enhancement of non-enzymatic defence. The lower phenolic content of sprayed plants could indicate a reduced demand for phenolic antioxidants where other components, such as SOD and POD, were more active, or consumption of phenolics during scavenging. The present data cannot distinguish between these explanations.

Ascorbic acid, soluble sugars and free amino acids were not significantly affected by any factor. The stability of ascorbic acid, despite large changes in APX activity, suggests that the ascorbate pool was maintained, in line with the central role of ascorbate and glutathione in redox homeostasis (Foyer & Noctor, 2005, 2011). The absence of responses in sugars and amino acids indicates that osmotic adjustment through these solutes was not a major component of the responses recorded here.

4.4 Overall interpretation and limitations

Taken together, the results show that foliar nano-biochar and sulphur modulate antioxidant responses in maize in different ways: sulphur mainly increased SOD and, under well-watered conditions, APX, whereas nano-biochar mainly increased POD under drought. Both sprays suppressed the drought-induced rise in APX and lowered phenolics. None of these changes was accompanied by lower H_2O_2 or MDA, so the data do not demonstrate improved oxidative status or drought tolerance. Nano-biochar has been described as having a larger surface area and higher adsorption capacity than conventional materials, although its risks and potential toxicity also need evaluation (Faizan *et al*., 2024), and the present study does not address either point.

The conclusions are limited to a single maize cultivar grown in pots, one dose of each foliar treatment, and one sampling time 15 days after spraying. Nano-biochar characteristics and the physiological basis of the observed responses were not examined, and not all components of the antioxidant network, such as glutathione, were measured. Growth, yield and field performance were not assessed here and require further testing, together with dose–response studies and measurements at several time points.

5. CONCLUSION

Foliar nano-biochar (3%) and sulphur (12 mg L^{-1}) modulated antioxidant responses in maize under well-watered and drought conditions, but in different ways. Drought increased H_2O_2, and this increase was confined to sulphur-treated plants. Sulphur increased SOD activity and, under well-watered conditions, APX activity. Nano-biochar increased POD activity under drought. Both sprays prevented the drought-induced rise in APX seen in unsprayed plants and reduced phenolic content, while MDA, ascorbic acid, soluble sugars and free amino acids were unaffected.

Neither spray reduced H_2O_2 or MDA, so these results do not show that foliar nano-biochar improves oxidative stress status or drought tolerance in maize, nor that it outperforms sulphur. The effects depended on the trait and on water availability. Future work should include growth and yield measurements, characterisation of the nano-biochar, dose–response and time-course studies, and field-scale evaluation.

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