

HrpNEA Seed Treatment Is Associated with Improved Stand Establishment, Nitrogen and Antioxidant Metabolism, and Grain Yield in Maize under Rainfed, Drought-Prone Field Conditions

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Abstract

Maize grown in the Brazilian second-crop (“safrinha”) season is frequently exposed to water limitation during grain filling, and seed treatment has been proposed to improve crop performance under such conditions. We evaluated seed treatment with a formulated product containing the elicitor protein HrpNEA (40 µg of formulated product seed⁻¹; 1% αβ harpin protein) in maize (hybrid DKB 390 PRO) grown under rainfed, drought-prone field conditions in a randomized complete block design, assessing 18 agronomic, physiological and biochemical traits spanning stand establishment, biomass partitioning, nitrogen metabolism, antioxidant responses, root system architecture and yield components. HrpNEA seed treatment was associated with a higher plant stand, greater root and shoot biomass and allocation to roots, higher nitrate reductase activity and leaf chlorophyll, increased superoxide dismutase and peroxidase activities, lower soluble protein and lipid peroxidation, and greater root length and surface area. With no change in kernel number, individual kernel weight and grain yield were 21.7% higher in treated plants. Thirteen of the 18 traits remained significant after false-discovery-rate correction; treated and control plots separated completely along the first principal component (57% of variance; exact within-block permutation test, $P = 0.031$), and all six physiological modules responded in a coordinated manner dominated by nitrate reductase activity, root length, biomass and antioxidant enzyme activity. A multiplicative decomposition of the yield response showed that kernel weight accounted for essentially the whole yield gain, whereas the higher stand was offset by an unmeasured compensatory factor, indicating that enhanced grain filling was the principal contributor. This systems-level covariation does not, however, establish the causal contribution of individual processes to yield, which could not be determined in this single-environment study.

1. Introduction

Maize (*Zea mays* L.) is one of the most widely cultivated and economically important crops worldwide, playing a central role in food, feed, and bioenergy production (Tanumihardjo et al. 2020; Erenstein et al. 2022). However, its productivity is highly sensitive to water availability, with drought stress negatively affecting plant growth and yield across all developmental stages (Gong et al. 2014). The reproductive phase, particularly from tassel emergence to early grain filling, is considered the most vulnerable period to water deficit, often resulting in significant yield losses (Bheemanahalli et al. 2022). The increasing frequency and intensity of drought events associated with climate change further exacerbate these challenges in rainfed production systems.

In Brazil, maize production has increasingly expanded into the second cropping season (safrinha), which is frequently characterized by irregular rainfall distribution and periods of water limitation during critical growth stages (Pinheiro et al. 2021; Lana et al. 2026). Under these conditions, crop performance depends not only on genetic tolerance but also on agronomic strategies that enhance plant resilience to environmental stress. Improving early establishment and maintaining physiological function during grain filling are key factors for stabilizing yield under such variable conditions.

Plant elicitors have been proposed as complementary tools to enhance crop responses to abiotic stress (Dong et al. 1999, 2005; Zhang et al. 2011; Jain et al. 2024). Among them, harpin proteins are a class of proteinaceous molecules secreted by plant-pathogenic bacteria that can activate plant defense and stress-related pathways (Wei et al. 1992; Choi et al. 2013). In this context, HrpNEA, a harpin-type elicitor protein derived from *Erwinia amylovora*, has been associated with the induction of physiological and biochemical responses linked to plant adaptation under stress conditions (Zhang et al. 2011). Previous studies have reported that harpin proteins can influence processes such as antioxidant activity, osmolyte accumulation, and root development, suggesting a potential role in modulating plant performance under abiotic stress (Zhang et al. 2011). Recent molecular work has begun to resolve the basis of these responses: harpin perception in tobacco requires the wall-associated kinase WAK2, a receptor-like kinase (Held et al. 2025); Hpa1 binds the plasma-membrane aquaporin PIP1;4 and enhances CO₂ transport and photosynthesis in *Arabidopsis* (Li et al. 2015); and different harpins activate distinct, partly salicylic-acid-independent, pathways in the C4 cereal sorghum (Patoliya et al. 2025).

Seed treatment represents a practical approach for delivering such elicitors, allowing early modulation of plant physiological responses during germination and seedling establishment (Rhaman 2025; Sharaf et al. 2026). Such treatments may induce a primed physiological state, in which plants exhibit enhanced responsiveness to subsequent stress conditions (Mauch-Mani et al. 2017; Douma et al. 2017). Under field conditions, particularly in stress-prone environments, improvements in early establishment and subsequent physiological adjustments may contribute to enhanced crop performance. However, despite increasing interest in elicitor-based technologies, there remains limited field-based evidence linking these responses to yield outcomes under realistic agronomic conditions. In Brazil, the regulatory framework for such products was consolidated by the National Bioinputs Law (Law 15,070/2024), which explicitly includes biostimulants and macromolecules among bioinputs (Barbosa et al. 2025), increasing the demand for field-validated evidence of efficacy.

Therefore, the objective of this study was to evaluate the effects of HrpNEA seed treatment on maize grown under rainfed, drought-prone safrinha conditions. Specifically, we investigated plant establishment, biomass partitioning, nitrogen metabolism, antioxidant-related responses, root system architecture, and yield components, with the aim of characterizing the associated physiological responses and their relationship to grain yield under water-limited field conditions.

2. Materials and Methods

Seed treatment was performed immediately before sowing with a commercial formulation containing 1% (w/w) αβ harpin protein (HrpNEA) as the active ingredient, applied at 40 µg of formulated product seed⁻¹ (equivalent to approximately 0.4 µg of harpin protein seed⁻¹). The product was diluted in water and applied to the seeds inside plastic bags, which were then shaken vigorously to ensure a uniform distribution over the seed surface; control seeds were treated with water alone following the same procedure. The hybrid DKB 390 PRO, extensively characterized as drought tolerant (Lavinsky et al. 2015; Souza et al. 2016; Avila et al. 2016), was sown at 0.70 m between rows with a target population of

65,000 plants ha⁻¹. The field design was a randomized complete block (RCB) with six replications; each experimental unit consisted of four 10-m rows. Fertilization followed the soil analysis (Table 1) and regional recommendations: 450 kg ha⁻¹ of NPK (08:14:16) at planting and 100 kg ha⁻¹ of nitrogen as urea at the V7 growth stage (seven expanded leaves).

Table 1
Chemical analysis of the soil in the 0–20 cm layer before establishment of the experiment.

pH	P-Me1	K ⁺	Ca ²⁺	Mg ²⁺	Al ³⁺	H + Al
Water	---- mg dm ⁻³ ----		----- cmolc dm ⁻³ -----			
6.1	11.0	78	2.8	1.1	0.00	2.2
SB	t	T	V	m	OM	
	----- cmolc dm ⁻³ -----		----- % -----		dag kg ⁻¹	
4.08	4.08	6.28	65	0	2.5	

Methodologies: pH > H2O (1:2.5) / Al, Ca, Mg > KCl 1N/ K,P > Mehlich (HCl 0,05N + H2SO4 0,025N).

The experiment was conducted during the second-crop season, known as safrinha, in the Cerrado biome, one of Brazil’s most important maize-producing regions, at the following geographical coordinates: 18°54’00” S, 48°09’38” W and 912 meters of altitude. According to the Köppen classification the climate of the site is Cwa, with an annual average precipitation of 1479 mm, with average temperature of 21.5 °C, minimum of 15.4 °C and maximum of 27.7 °C. Figure 1 shows the meteorological elements observed during the experiment.

2.1 Plant Stand, Plant Height, and Chlorophyll Content

Plant height was assessed at the V8 growth stage by measuring the distance (cm) from the soil surface to the insertion point of the last fully expanded leaf. Measurements were taken using a measuring tape on ten consecutive plants per plot.

Chlorophyll content (SPAD index) was estimated using a SPAD-502 chlorophyll meter (Bullock and Anderson 1998). Measurements were taken on ten plants per plot, with three readings collected from the central portion of the leaf blade, avoiding the midrib and major veins. Readings were performed at two growth stages: at V4, on the most recently fully expanded leaf; and at VT (flowering), on the ear leaf.

2.2 Nitrate reductase activity and protein content

Nitrate reductase (NR) activity was determined in ear leaves collected at the R2 growth stage (blister; early kernel formation, after kernel number stabilization) from three plants per plot, following Cataldo et

al. (1975), and is expressed as $\mu\text{mol NO}_2^- \text{ g}^{-1} \text{ FW h}^{-1}$. Total soluble protein content was determined according to Bradford (1976) and is expressed as $\text{mg g}^{-1} \text{ FW}$.

2.3 Antioxidant enzymes activity

The assessment of antioxidant enzymes activity was conducted during the R2 growth stage. Leaf samples were collected between 08:00 and 10:00 in the morning, a period when the enzymes exhibit heightened activity. These samples were placed in plastic bags and wrapped in aluminum foil. The leaves were ground in liquid nitrogen, followed by the addition of 10 mL of potassium phosphate buffer 0.1 M pH 6.8 (Kar and Mishra 1976). Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined following Beauchamp and Fridovich (1971) and is expressed as U mg^{-1} protein, one unit being the amount of enzyme that inhibits nitroblue tetrazolium photoreduction by 50%. Peroxidase (POD, EC 1.11.1.7) activity was determined according to Teisseire and Guy (2000) and is expressed as $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ protein. Lipid peroxidation was assessed as malondialdehyde (MDA) content following Heath and Packer (1968). Proline content was determined by the method of Bates et al. (1973).

2.4 Root Morphology

The root morphology was determined in two plants per plot collected at V4 growth stage. Soil blocks were extracted using a square-edged cutting shovel. After collection, the plants were separated into root and aboveground parts. The roots were placed into plastic trays and carefully washed using meshes to prevent material loss. The root morphology analysis was performed using the Winrhizo® software, version 4.1, coupled with an Epson XL 10000 scanner (Magalhães et al. 2011). The root parameters retrieved were total length, RTL (cm) and surface area, RSA (cm^2). Following the images capturing, the roots and shoot were dried on paper towels and stored in paper bags for dry matter determination.

2.5 Root and shoot dry matter

The roots and shoots were dried in a forced ventilation oven at 65°C until they reached a constant mass. Weighing was performed using a digital scale with a precision of 0.01 grams.

2.6 Yield and yield components

Yield components and grain productivity were evaluated on an experimental unit area of 9 m^2 per replication. At harvest, grain moisture content was determined, and grain yield was calculated after adjustment to a standard moisture content of 13% (0.13 g g^{-1}). Grain mass was measured using a digital scale with a precision of 0.01 g. Yield components were manually assessed from 10 ears per replication. The evaluated traits included the number of rows per ear (NRE), number of kernels per row (NKR), number of kernels per ear (NKE), and thousand-kernel weight, from which individual kernel weight (KWT) was calculated. Final plant population at harvest and the number of ears per plant were not recorded.

2.7 Statistical analysis

Each trait was analysed with a randomized complete block ANOVA (treatment + block). With two treatments this F-test is identical to a block-paired t-test, so no post hoc multiple-comparison procedure

was required; normality of the within-block differences was verified with the Shapiro-Wilk test (all $P > 0.05$). Because P-values alone convey little about the magnitude of effects, each trait is also reported as the mean within-block difference and percentage change relative to the control with 95% confidence intervals, and as a standardized paired effect size (Cohen's d_z ; Lakens 2013). Raw P-values were corrected for multiple testing across traits with the Benjamini–Hochberg false-discovery-rate (FDR) procedure (Benjamini and Hochberg 1995), with significance set at $q < 0.05$. Three of the 21 recorded variables (total dry matter, root:shoot ratio and kernels per ear) are exact arithmetic functions of other measured variables; they are reported for agronomic interpretation but were excluded from the multivariate analyses and from the primary FDR family, which therefore comprised the 18 independently measured traits (results for all 21 variables are given in Table S1). All analyses were performed in Python (pandas, SciPy, statsmodels, scikit-learn; Pedregosa et al. 2011; Seabold and Perktold 2010), and the scripts reproducing every statistic and figure are archived in the associated data repository (see Data and Code availability).

To evaluate whether the treatment effect was coordinated across physiological processes rather than confined to isolated traits, a systems-level analysis was carried out in four steps. (i) The 18 measured traits were assigned a priori to six physiological modules (growth and biomass; photosynthetic status; nitrogen and protein metabolism; oxidative stress system; root architecture; yield components) on the basis of their biological function. (ii) Principal Component Analysis (PCA) was performed on the standardized (z-scored) trait matrix. Because the number of traits exceeded the number of experimental units (12), PCA was used as a descriptive ordination, and the significance of the multivariate treatment effect was instead assessed with an exact permutation test that respects the block structure: treatment labels were exchanged within blocks in all $2^6 = 64$ possible ways and the squared Euclidean distance between the two treatment centroids in z-space was recomputed for each relabelling (Anderson 2001; Good 2005). The proportions of PC1 and PC2 score variance attributable to treatment and to block were quantified by one-way ANOVA of the scores. (iii) For each module, a module eigentrait was computed as the first principal component of its member traits, analogous to the module eigengene of weighted correlation network analysis (Langfelder and Horvath 2008), and the six eigentraits were tested for a treatment effect with block-paired t-tests and FDR correction, thereby reducing 18 correlated tests to six module-level tests. Pearson correlations among traits and among module eigentraits were computed both on the raw data and after centring each treatment group on its own mean (treatment-centred correlations), to distinguish covariation that merely reflects the common treatment shift from covariation among plots within treatments. (iv) Finally, the grain-yield response was decomposed multiplicatively into its components: for each block, $\ln(\text{GY}_{\text{HrpNEA}}/\text{GY}_{\text{control}})$ was partitioned into the log-ratios of plant stand, kernels per ear and individual kernel weight, the remainder representing unmeasured components (final harvest population, ears per plant and ear-sampling error); yield was also predicted from the product of stand, kernels per ear and kernel weight and compared with the observed plot yield.

This study did not involve human participants or animals; therefore, no ethics approval was required.

3. Results

3.1 Weather conditions throughout the growing season

The weather conditions were favorable for crop growth and development throughout the vegetative phase, with well-distributed rainfall during this period. The minimum and maximum temperatures observed during the crop cycle were 11.3°C and 32.8°C respectively (Fig. 1). The accumulated precipitation was 327.6 mm during the vegetative phase and 95.0 mm during the reproductive phase, totaling 422.6 mm until harvest (Fig. 1). However, during the grain filling there were 37 days of water restriction, which extended from R1 (silking) to R5 (dent) growth stages (Ritchie et al. 1986).

3.2 Germination, emergence, and plant stand

Differences in stand establishment were evident following HrpNEA seed treatment. Plant density, expressed as the number of plants per linear meter, was significantly higher than in the control, with a 13.7% increase (3.65 vs 4.15 plants m⁻¹, equivalent to ≈ 52,100 and ≈ 59,300 plants ha⁻¹, i.e., 80% and 91% of the target population of 65,000 plants ha⁻¹; Fig. 2a). In contrast, plant height was not significantly affected (Fig. 2b), indicating that the response was specific to early establishment processes rather than a general stimulation of vegetative growth.

3.3 Nitrate Reductase, Total Protein and Chlorophyll Content

Coordinated changes in nitrogen metabolism and photosynthetic traits were observed in maize following HrpNEA seed treatment. Nitrate reductase (NR) activity, a key regulatory step in nitrate assimilation, was 66.1% higher in treated plants than in the control (Fig. 3a). This response is consistent with a treatment-mediated modulation of nitrogen assimilation processes and may reflect alterations in nitrate sensing or downstream regulatory pathways, including those involving nitric oxide. Notably, the increase in NR activity occurred alongside an 11.1% reduction in total soluble protein content (Fig. 3b), suggesting a redistribution of nitrogen towards specific functional pools rather than a generalized increase in protein accumulation.

Consistent adjustments were also detected in photosynthetic traits, with higher chlorophyll content in treated plants at V4, as indicated by a significant 10.8% increase in relative SPAD values at that stage; a smaller increase of 8.1% at VT was not statistically significant (Fig. 3c,d). Given the close coupling between nitrogen availability and chlorophyll biosynthesis, this pattern aligns with the observed changes in NR activity and suggests an adjustment of photosynthetic capacity. Together, these responses point to a regulated reconfiguration of nitrogen use and photosynthetic traits that may enhance metabolic flexibility under water-limited field conditions.

3.4 Biomass Partitioning

Changes in biomass partitioning were observed following HrpNEA treatment (Fig. 4). Root dry matter (RDM) increased by 47.0% (Fig. 4a), accompanied by more moderate increases in shoot dry matter

(SDM; 13.3%) and total dry matter (TDM; 17.0%) (Fig. 4b,c). The resulting 30.1% increase in the RDM/SDM ratio (Fig. 4d) indicates a shift in assimilate allocation towards below-ground tissues, rather than a uniform stimulation of biomass accumulation, consistent with enhanced investment in root system development.

3.5 Antioxidative Stress

Modulation of antioxidant defense systems was observed following HrpNEA treatment under stress conditions. Drought-associated oxidative stress is characterized by the accumulation of reactive oxygen species (ROS), which can lead to cellular dysfunction if not efficiently detoxified. Superoxide dismutase (SOD) activity increased markedly, reaching levels 73.9% higher than in the control (Fig. 5b), while peroxidase (POD) activity also showed a significant increase of 19.4% (Fig. 5c). These enzymatic changes were accompanied by a 17.4% reduction in malondialdehyde (MDA) content (Fig. 5a), a marker of lipid peroxidation, consistent with enhanced ROS-scavenging capacity. The coordinated upregulation of SOD and POD activity may facilitate the sequential detoxification of superoxide radicals and hydrogen peroxide, thereby limiting oxidative damage.

In parallel, proline content showed a non-significant increase of 8.0% relative to the control (Fig. 5d). Although proline accumulation is commonly associated with abiotic stress responses, the lack of statistical significance means this trend should be interpreted with caution. Together, these observations suggest that HrpNEA treatment is associated with improved management of oxidative stress, through higher SOD and POD activity and reduced lipid peroxidation, with only limited evidence for an osmotic-adjustment component under these conditions.

3.6 Root Morphology

Substantial changes in root system architecture were detected in plants associated with HrpNEA seed treatment. Total root length increased by 74.1%, and root surface area by 19.7%, relative to the control (Fig. 6a,b). These responses indicate greater root elongation and branching, traits that expand the explored soil volume and are relevant for resource acquisition under spatially and temporally variable water and nutrient availability, such as those characteristics of the safrinha season.

The observed increases in root length and surface area coincide with shifts in biomass partitioning and stress-related physiological traits reported here, including increased root dry matter accumulation and altered antioxidant enzyme activities. Together, these coordinated responses suggest a redistribution of assimilates towards belowground structures and processes commonly associated with stress acclimation. Such adjustments may reflect enhanced developmental and metabolic plasticity under water-limited field conditions, without implying a uniform stimulation of growth across plant organs.

3.7 Yield Components and Grain Production

No significant differences were detected in the number of rows per ear, kernels per row, or total kernel number per ear across treatments (Fig. 7a-c), indicating that the determinants of kernel set were not altered. In contrast, a significant increase in individual kernel weight was observed (Fig. 7d), pointing to an effect on processes operating during the grain-filling phase rather than on reproductive development per se. Grain filling represents a key yield-determining stage under stress-prone environments such as the safrinha season, and variation in kernel weight often reflects post-anthesis physiological conditions.

The increase in kernel weight occurred in parallel with earlier changes in photosynthetic traits, root system architecture, antioxidant activity, and stand establishment, suggesting coordination between source capacity and sink demand. Consistent with this pattern, total grain yield was 21.6% higher than the control (Fig. 8), closely matching the gain in individual kernel weight (+ 21.5% and + 21.6%); the higher plant stand did not translate into a proportional increase in area yield, indicating that enhanced grain filling was the dominant contributor to the yield response. Together, these responses indicate an adjustment of source-sink relationships that favors kernel filling under field conditions characterized by variable water availability, without evidence of altered kernel number. This pattern highlights the contribution of physiological and developmental plasticity to yield performance under stress.

3.8 Integrated Systems-Level Analysis

To evaluate whether the responses reported above (Sections 3.2–3.7) reflect a coordinated, systems-level response to HrpNEA seed treatment rather than independent effects, the 18 measured traits were analysed jointly (Fig. S1; Tables S1 and S5-S8). Thirteen of the 18 measured traits (15 of 21 when the three derived variables are included) remained significant after FDR correction ($q < 0.05$), with paired effect sizes ranging from $d_z = 1.3$ (MDA) to 4.8 (grain yield), and the 95% confidence interval of the percentage change excluded zero for every one of them (Fig. S1; Table S8). The within-block differences of all traits were consistent with normality (Shapiro-Wilk $P \geq 0.07$).

PCA of the 18-trait matrix showed that the first principal component (PC1) explained 57.2% of total variance (PC1 + PC2 = 70.1%) and separated the six HrpNEA-treated plots from the six control plots completely (Fig. S2a); treatment accounted for 97% of the variance in PC1 scores, whereas block accounted for 69% of the variance in PC2 scores (12.9% of total variance), identifying PC2 as a spatial (block) axis. Nitrate reductase activity, root length, superoxide dismutase activity, shoot dry matter and kernel weight had the largest PC1 loadings (Table S5). Including the three derived variables (PC1 = 56.5%) or removing block means before ordination (PC1 = 71.8% of within-block variance) did not alter this pattern (Table S5). The exact within-block permutation test confirmed that a separation of this magnitude is unlikely to arise by chance ($P = 0.031$, the smallest two-sided value attainable with six blocks; Fig. S2b).

At the module level, all six eigentraits differed between treatments (block-paired t-tests, $q \leq 0.011$; Table S6, Fig. S4a), with the largest standardized effects in nitrogen and protein metabolism ($d_z = 4.8$), root architecture (3.7), growth and biomass (3.6) and yield components (3.4), and the smallest in

photosynthetic status (1.6). Module eigentraits were strongly inter-correlated across the 12 plots ($r = 0.63\text{--}0.95$; Fig. S4b), but these correlations were generated almost entirely by the common treatment shift: after centring each treatment on its own mean, the mean absolute correlation among the 18 traits fell from 0.52 to 0.25, no trait pair remained significant after FDR correction, and within-module correlations were no stronger than between-module correlations (mean $|r| = 0.49$ vs 0.52 on the raw data; Fig. S3, Fig. S4c). The coordinated response is therefore a property of the treatment contrast itself rather than of the covariation among plots within a treatment.

The multiplicative decomposition of the yield response (Fig. S5, Table S7) showed that the 21.7% yield gain (95% CI 16–28%) was matched almost exactly by the gain in individual kernel weight (+ 21.7%, CI 12–32%), whereas the gains in plant stand (+ 13.8%, CI 4–24%) and kernels per ear (+ 5.4%, CI -5 to 17%) were offset by an unaccounted factor of -16.6% (CI -25 to -7%). Consistently, yield predicted from the product of stand, kernels per ear and kernel weight matched the observed yield in the control plots (observed/predicted = 1.02) but over-predicted it in the treated plots (0.85), indicating that the early stand advantage did not persist proportionally to harvest, or that ears per plant declined at the higher density.

4. Discussion

The present study evaluated the responses of maize to HrpNEA seed treatment under water-limited field conditions typical of the safrinha season. Across developmental stages, treated plants exhibited coordinated changes in stand establishment, biomass partitioning, physiological traits, and yield components. These responses were largely process-specific and occurred without alterations in kernel number, suggesting that yield differences were associated with establishment efficiency and grain filling rather than changes in reproductive determination. Comparable elicitor and biostimulant-based treatments in maize have also been associated with improved performance under water deficit, including chitosan application and seed priming with plant-derived extracts (Almeida et al. 2019; Wazeer et al. 2025), supporting the broader relevance of seed-based elicitor strategies in maize and aligning with the rapid recent growth of biostimulant research under drought stress (Liaqat et al. 2026).

4.1 Stand establishment and early development

Stand establishment is a key determinant of yield potential, particularly under stress-prone environments where early-stage constraints can limit crop performance. The increase in plant density observed following HrpNEA seed treatment indicates an effect on germination and/or early seedling establishment under field conditions.

Given that water availability during emergence can be variable in the safrinha season, improved stand establishment may reflect enhanced seed vigor or early metabolic activity, although these processes were not directly assessed in this study. Although maize yield is sensitive to realized plant population (Milander et al. 2017), the yield gain here closely matched the increase in individual kernel weight and

was not proportionally larger, so the net contribution of the higher stand density to area yield could not be isolated (Section 4.6); early establishment nonetheless remains agronomically relevant under rainfed conditions. This relevance is underscored by recent second-crop maize seasons in the Brazilian Cerrado, where water deficits during reproductive development and grain filling have reduced yield potential in several major producing areas (CONAB 2026).

4.2 Nitrogen metabolism and photosynthetic traits

Changes in nitrate reductase activity and chlorophyll content indicate adjustments in nitrogen assimilation and photosynthetic-related traits in treated plants. The increase in nitrate reductase activity suggests altered regulation of nitrate assimilation, while higher chlorophyll content is consistent with adjustments in photosynthetic capacity.

However, as nitrogen partitioning and photosynthetic rates were not directly measured, these responses should be interpreted as indicative of altered metabolic status rather than as evidence of increased carbon assimilation per se. The observed patterns are consistent with previous reports of drought-induced adjustments in nitrogen metabolism and primary metabolite profiles, as well as ABA-mediated regulation of stomatal function and water-use efficiency (Silvente et al. 2012; Kuromori et al. 2018). Further studies measuring gas exchange, nitrogen allocation, and metabolite fluxes will be required to clarify the underlying regulatory mechanisms.

4.3 Biomass partitioning and carbon allocation

HrpNEA seed treatment was associated with changes in biomass allocation, particularly an increased investment in root tissues relative to shoots. The higher root-to-shoot ratio reflects a shift in assimilate partitioning toward belowground structures, a response commonly observed under water-limited conditions and associated with strategies to sustain water-use efficiency (Delatorre-Herrera et al. 2010).

Such allocation patterns may enhance the capacity for soil exploration and resource acquisition; however, root water uptake was not directly measured in this study. Therefore, the observed changes are best interpreted as indicative of altered allocation strategies rather than direct evidence of improved water acquisition efficiency. These adjustments likely contributed to maintaining plant function during later growth stages.

4.4 Antioxidant responses and osmotic adjustment

Modulation of antioxidant enzyme activity and osmolyte accumulation was observed in treated plants under stress conditions. Increased activity of superoxide dismutase and peroxidase, together with reduced lipid peroxidation, indicates changes in oxidative status, consistent with the well-established role of these enzymes in scavenging reactive oxygen species and limiting membrane damage under abiotic stress (Alscher 2002; Gill and Tuteja 2010; Niu and Xiang 2018; Batool et al. 2022). The non-significant trend toward higher proline content is consistent with, but does not by itself establish, adjustments in osmotic regulation, a response frequently reported alongside changes in antioxidant

enzyme activity and lipid peroxidation under water and salt stresses in other species (Koca et al. 2007; Hatzig et al. 2014; Harsh et al. 2016).

While these responses are consistent with commonly reported stress-associated biochemical changes, including those of complementary antioxidant enzymes such as ascorbate peroxidase and catalase that were not assessed here (Sofo et al. 2015), they do not directly demonstrate improved stress tolerance. Instead, they indicate that HrpNEA seed treatment is associated with alterations in biochemical processes related to stress response. The extent to which these changes contribute to improved field performance remains to be further investigated.

4.5 Root system architecture

Substantial increases in root length and surface area were observed in treated plants, indicating pronounced changes in root system architecture. These traits are associated with increased soil exploration and may be advantageous under heterogeneous water availability, consistent with root ideotypes proposed to optimize water and nutrient acquisition under resource-limited conditions (Lynch 2013; Kaur et al. 2026).

However, as soil moisture dynamics and water uptake were not directly assessed, the functional implications of these changes should be interpreted with caution. The observed root responses, together with shifts in biomass partitioning, suggest coordinated developmental adjustments rather than uniform stimulation of growth.

4.6 Yield components and grain yield

Yield component analysis indicated that kernel numbers were not affected by HrpNEA seed treatment, whereas individual kernel weight increased. This pattern suggests that the primary effects of the treatment occurred during the grain-filling phase rather than during reproductive establishment, consistent with the recognized separation between processes determining kernel number around silking and those determining kernel weight during grain filling (Borrás et al. 2004).

The increase in total grain yield closely tracked the increase in individual kernel weight (both + 21.7% in the paired analysis), indicating that enhanced grain filling was the principal contributor. Notably, the higher plant stand did not translate into a proportional increase in area yield: a multiplicative combination of the stand (+ 13.8%), kernel-number (+ 5.4%) and kernel-weight (+ 21.7%) gains would predict a yield increase of roughly 45%, twice that observed, and the yield-component product over-predicted the observed yield of treated plots by about 15% while matching the control plots within 2% (Section 3.8; Fig. S5). This points to density compensation, i.e. a reduction in ears per plant or in the fraction of established plants surviving to harvest as population increased from $\approx 52,000$ to $\approx 59,000$ plants ha^{-1} , or to the early stand advantage not persisting to harvest; because final harvest population and ears per plant were not recorded, the net contribution of stand density cannot be isolated. Given the absence of changes in kernel number, the yield improvement appears to be associated primarily with post-anthesis grain-filling processes, in which kernel weight is set by assimilate supply and stem-reserve

remobilization during the effective grain-filling period (Borrás et al. 2004; Chazarreta et al. 2025), rather than with reproductive determination or plant density per se, although these processes were not directly quantified.

4.7 Systems-level integration

The module-by-module patterns discussed above (Sections 4.1–4.6) were also evaluated jointly (Section 3.8). Thirteen of the 18 measured traits remained significant after Benjamini-Hochberg correction, all six physiological modules responded to the treatment when summarized as module eigentraits, and Principal Component Analysis showed that a single axis (PC1), dominated by nitrate reductase activity, root length, superoxide dismutase activity, shoot dry matter and kernel weight, explained 57% of total variance and separated treated from control plots completely, a separation that an exact within-block permutation test showed to be unlikely under the null hypothesis ($P = 0.031$). This indicates that the nitrogen-metabolism, biomass, root and antioxidant responses did not vary independently across the 12 plots but changed together, consistent with a single coordinated physiological response to the treatment rather than several unrelated effects.

The second axis (PC2, 12.9% of variance) was unrelated to treatment and was largely explained by block (69% of its score variance), so that it most likely reflects spatial heterogeneity in soil or water availability that is independent of the treatment effect. The separation of a treatment-associated axis (PC1) from a block-associated axis (PC2) supports the interpretation that the coordinated response is attributable to HrpNEA seed treatment rather than to spatial variability within the experimental area. Two caveats follow from the treatment-centred analysis, however. First, the strong correlations among traits and modules across the 12 plots are almost entirely generated by the treatment contrast: once each treatment group is centred on its own mean, the residual correlations are weak, none survives FDR correction, and traits within a module are no more correlated than traits from different modules. The “coordination” is therefore a statement about the direction and consistency of the treatment effect across processes, not evidence of regulatory coupling among processes within plants. Second, as with the individual physiological responses, this systems-level association does not establish the specific regulatory or causal links between modules (e.g., whether nitrogen metabolism changes preceded and drove biomass allocation, or vice versa), which would require targeted mechanistic study with larger numbers of experimental units.

4.8 Proposed physiological mechanism

Building on the module-level and systems-level associations described above, a tentative physiological cascade is proposed to organize the observed responses into a coherent sequence (Fig. 9). This model draws on the general mechanism of harpin perception and signaling described in the elicitor literature and is not itself tested by the present dataset; it is offered as a working hypothesis to guide future mechanistic studies. Harpin-type elicitors, including HrpNEA, are extracellular proteins secreted by plant-pathogenic bacteria through the type III secretion system and are perceived at or near the plant plasma membrane, where they activate early defence-signaling responses (Alfano and Collmer 2004; Wei et al.

1992; Choi et al. 2013). These responses may include transient ROS production and MAPK activation, followed by downstream modulation of defence and growth-related hormone pathways, including salicylic acid, ethylene, and gibberellin signaling, although the relative contribution of each pathway varies among harpin proteins and plant species (Baker et al. 1993; Dong et al. 1999; Desikan et al. 2001; Li et al. 2014). Recent molecular work supports several of these steps: the harpin-induced hypersensitive response in tobacco requires the wall-associated kinase WAK2, a receptor-like kinase candidate for harpin perception (Held et al. 2025); Hpa1 binds the plasma-membrane aquaporin PIP1;4, increasing CO₂ transport, mesophyll conductance and photosynthesis (Li et al. 2015); and in the C4 cereal sorghum, HrpZ and Hpa1 activate distinct salicylic-acid-dependent and independent pathways (Patoliya et al. 2025), indicating that the signalling route is harpin and species-specific.

None of these early signaling intermediates were measured in the present study and are therefore represented as inferred, rather than demonstrated, steps in Fig. 9. The magnitude and breadth of the physiological responses reported here are broadly consistent with harpin-elicitor effects described in other crops, including enhanced growth and disease resistance in transgenic rapeseed expressing a harpin-encoding gene (Huo et al. 2010), hrpN-induced growth stimulation in tobacco (Jang et al. 2006; Sohn et al. 2007), and harpin-induced innate immune priming in strawberry (Scariotto et al. 2021), although direct comparison is limited by differences in species, delivery method, and the traits measured.

Within this framework, the marked increase in nitrate reductase activity may constitute a mechanistic link between treatment-induced signaling and the antioxidant and nitrogen-status responses observed here. Nitrate reductase-derived nitric oxide is recognized as an important regulator of ROS homeostasis and antioxidant defenses during plant stress (Gupta et al. 2025), consistent with simultaneous increases in SOD and POD activities and reduction in MDA content in treated plants. Nevertheless, direct quantification of nitric oxide or genetic disruption of the NR-NO pathway would be required to confirm this mechanism (Section 3.5). Downstream, the shift in biomass allocation toward roots (Section 3.4) and the sustained chlorophyll status (Section 3.3) may reflect a broader reallocation of resources that, together with the independent early-establishment effect on stand (Section 3.2), converges on the improvements in kernel weight and grain yield described in Section 3.7. This proposed sequence is consistent with the coordinated, single-axis pattern identified by the systems-level analysis (Section 4.7), but the causal ordering and regulatory links among these processes remain to be established through targeted physiological and molecular studies. Nitric oxide is also increasingly recognized as an integral element of priming-induced tolerance and stress memory at both the seed and the vegetative stage (Fejes et al. 2025), and drought stress memory in maize has been linked to epigenetic and metabolic adjustments (proline biosynthesis, carbon-nitrogen reallocation) that are amenable to field-applicable seed-priming strategies (Quyoom et al. 2026); the sustained differences in nitrogen and antioxidant status observed here at R2, months after seed treatment, are compatible with such a primed state, although no memory-related mark was measured.

Taken together, the results indicate that HrpNEA seed treatment is associated with a set of coordinated responses spanning establishment, physiological traits, and yield components under water-limited field conditions. These responses appear to reflect adjustments in plant function across developmental stages rather than a uniform stimulation of growth. Importantly, the yield gain closely matched the increase in individual kernel weight, so enhanced grain filling rather than plant density appears to have been the principal contributor to productivity under the conditions evaluated; the net contribution of the higher stand density could not be isolated in this design. Other responses, including changes in metabolism, root architecture, and stress-related traits, may have contributed to maintaining plant performance during later stages, although their relative importance cannot be disentangled in the present study.

Overall, the findings highlight the potential of seed-treatment approaches to influence crop performance under variable environmental conditions, while also underscoring the need for further studies to clarify the mechanisms underlying these responses and their consistency across environments. In particular, because the study comprised a single rain-fed environment without a well-watered comparison, it cannot establish whether these responses are specific to water limitation; a water-level contrast, together with multiple environments and genotypes, would be required to attribute them to drought mitigation. For context, a meta-analysis of 180 field studies estimated an average biostimulant yield gain of 17.9% (about 17% for seed treatments), with larger responses under water scarcity and in suboptimal soils (Li et al. 2022); the 21.7% gain observed here therefore lies in the upper part of the range reported for field biostimulants, and is comparable to the 9.5–16.9% gains reported for a harpin-based product in maize under reduced fertilization (Huang et al. 2026) and to the delayed leaf senescence and improved yield obtained with a plant-derived biostimulant under reproductive-stage drought (Kanojia et al. 2026). Such comparisons also underline that single-site responses of this magnitude should be confirmed across environments before agronomic recommendations are made.

5. Conclusions

The results of this study indicate that HrpNEA seed treatment is associated with a range of physiological and developmental responses in maize grown under water-limited field conditions typical of the safrinha season. The most consistent effects were observed in stand establishment and individual kernel weight, which together contributed to the increase in grain yield.

Changes in nitrogen metabolism, chlorophyll content, antioxidant-related traits, biomass partitioning, and root system architecture were also detected, suggesting adjustments in plant physiological status across developmental stages. However, as these processes were not directly linked to specific functional outcomes, their contribution to yield performance should be interpreted with caution.

An integrated, systems-level analysis further supported this interpretation: after correction for multiple testing, 13 of the 18 measured traits remained statistically significant; all six physiological modules responded when summarized as module eigentraits; and a multivariate ordination showed that nitrogen

metabolism, biomass accumulation, root architecture and antioxidant-related traits covaried along a single dominant axis that completely separated treated from control plots (exact permutation $P = 0.031$). This indicates that HrpNEA seed treatment elicited a broad, coordinated physiological response rather than a set of unrelated changes, even though the present design cannot establish mechanistic or causal relationships among these responses, and the covariation reflects the consistency of the treatment effect rather than coupling among processes within treatments.

Importantly, yield responses occurred without changes in kernel number, indicating that improvements were primarily associated with post-anthesis processes and plant establishment rather than reproductive determination. A multiplicative decomposition showed that the gain in individual kernel weight accounted for the entire 21.7% yield gain, whereas the 13.8% higher stand was offset by an unmeasured compensatory factor, so that enhanced grain filling rather than plant density was the principal contributor; the design cannot fully isolate the contribution of stand density.

Overall, these findings suggest that HrpNEA seed treatment may represent a practical approach to support maize performance under variable water availability, particularly by improving early establishment and grain filling. Further studies across environments and management conditions will be required to assess the consistency of these responses and to better understand the underlying mechanisms.

Declarations

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Consent to participate Not applicable.

Consent for publication Not applicable.

Data availability The raw data supporting this study are openly available in the Zenodo repository [<https://doi.org/10.5281/zenodo.22770335>].

Code availability The analysis code that reproduces all statistics and figures (analysis.py for the trait-by-trait statistics, PCA and correlation matrix; systems_analysis.py for the effect sizes, permutation test, module eigentrains and yield decomposition) is available in the same repository (see Data availability).

Author Contributions Fernando Rodrigo de Oliveira Cantão contributed to the conceptualization of the study, conducted the experiment and data collection, performed the data analysis, and wrote and reviewed the manuscript. Antônio Carlos de Oliveira contributed to the data analysis. Evaristo Mauro de Castro, Rogerio Falleiros Carvalho, and Paulo César Magalhães reviewed the manuscript. All authors read and approved of the final manuscript.

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Figures

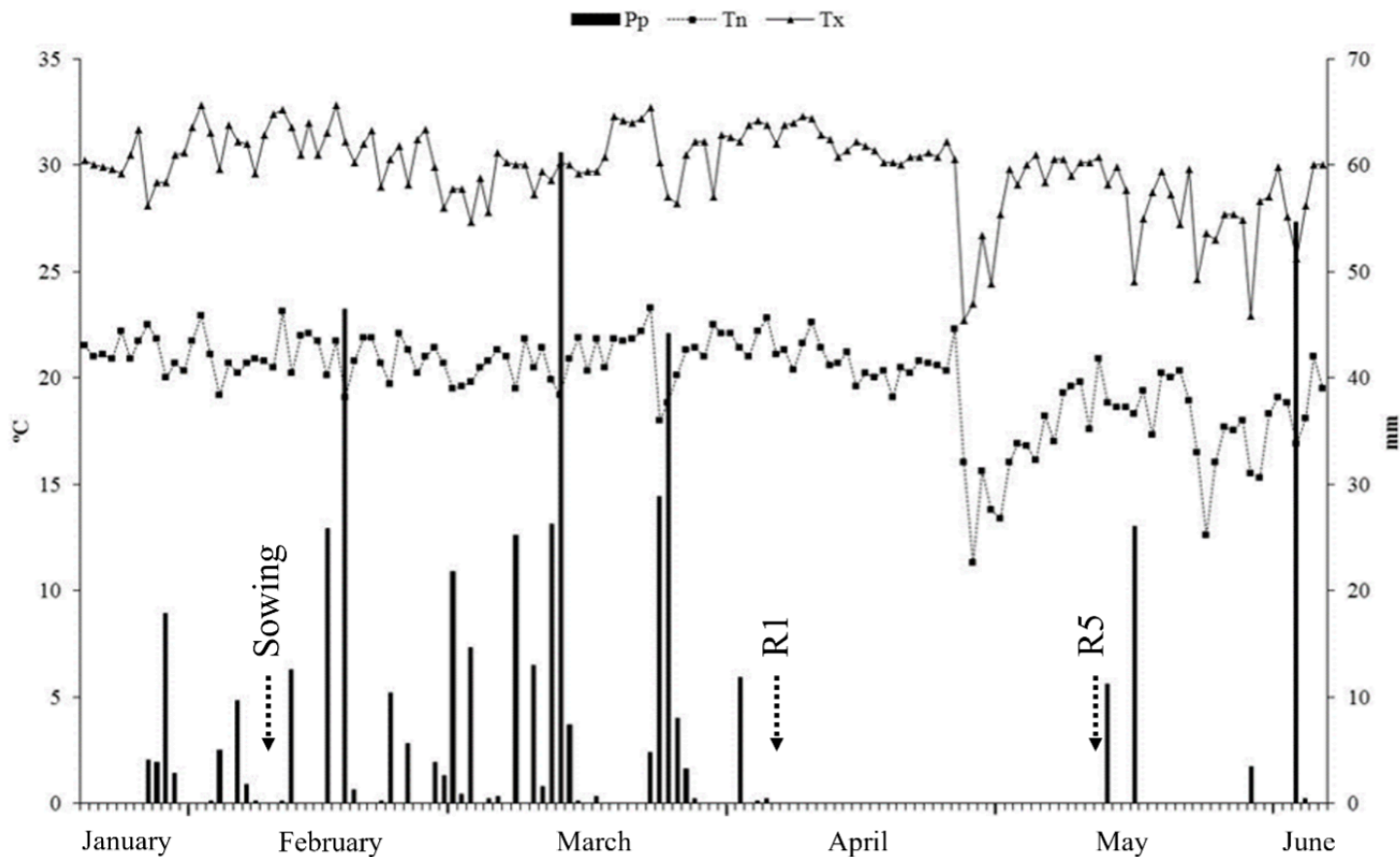


Figure 1

Weather conditions (Pp, precipitation; Tn, minimum temperature and Tx, maximum temperature), observed during the experiment period. Sowing date and the, R1 and R5 growth stages are indicated.

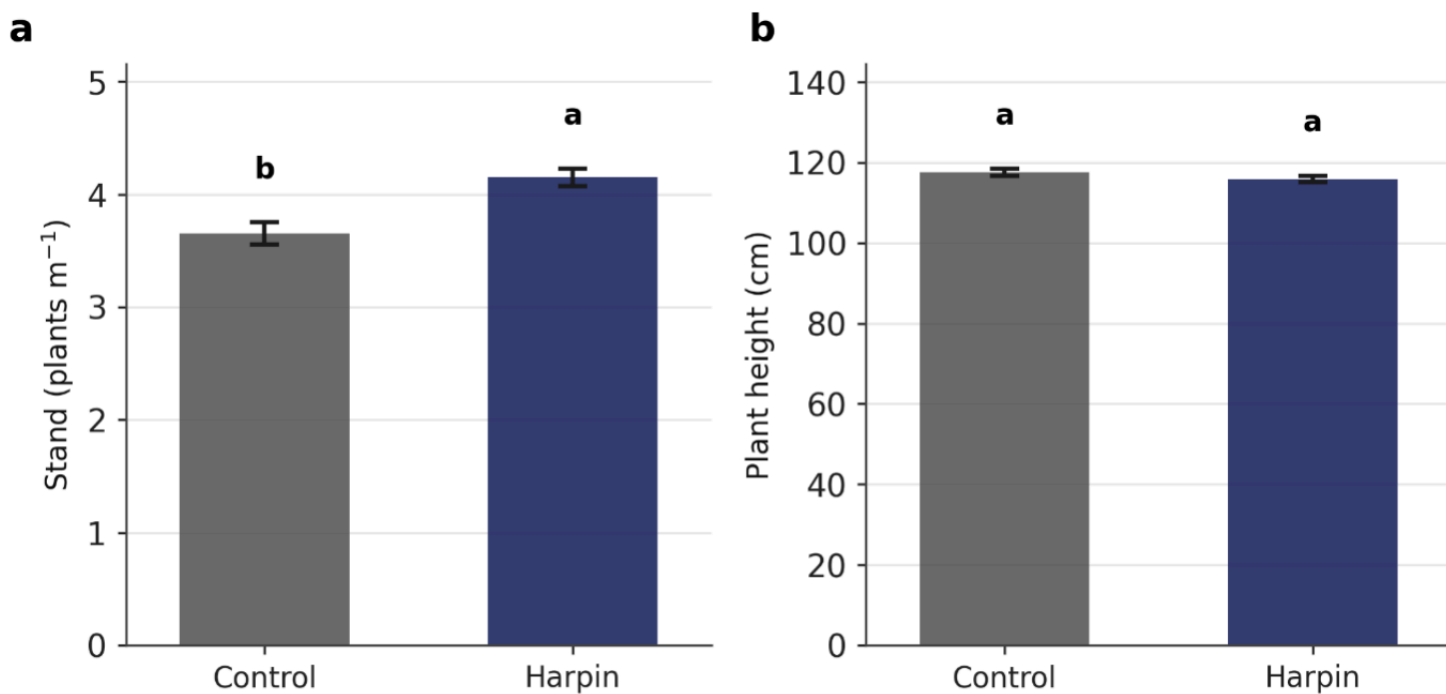


Figure 2

Effect of HrpNEA applied in seed treatment on: (a) stand (number of plants per linear meter); (b) plant height (cm). Means followed by the same letter do not differ (randomized complete block ANOVA, $P \leq 0.05$, equivalent to a block-paired t-test; $n = 6$ blocks).

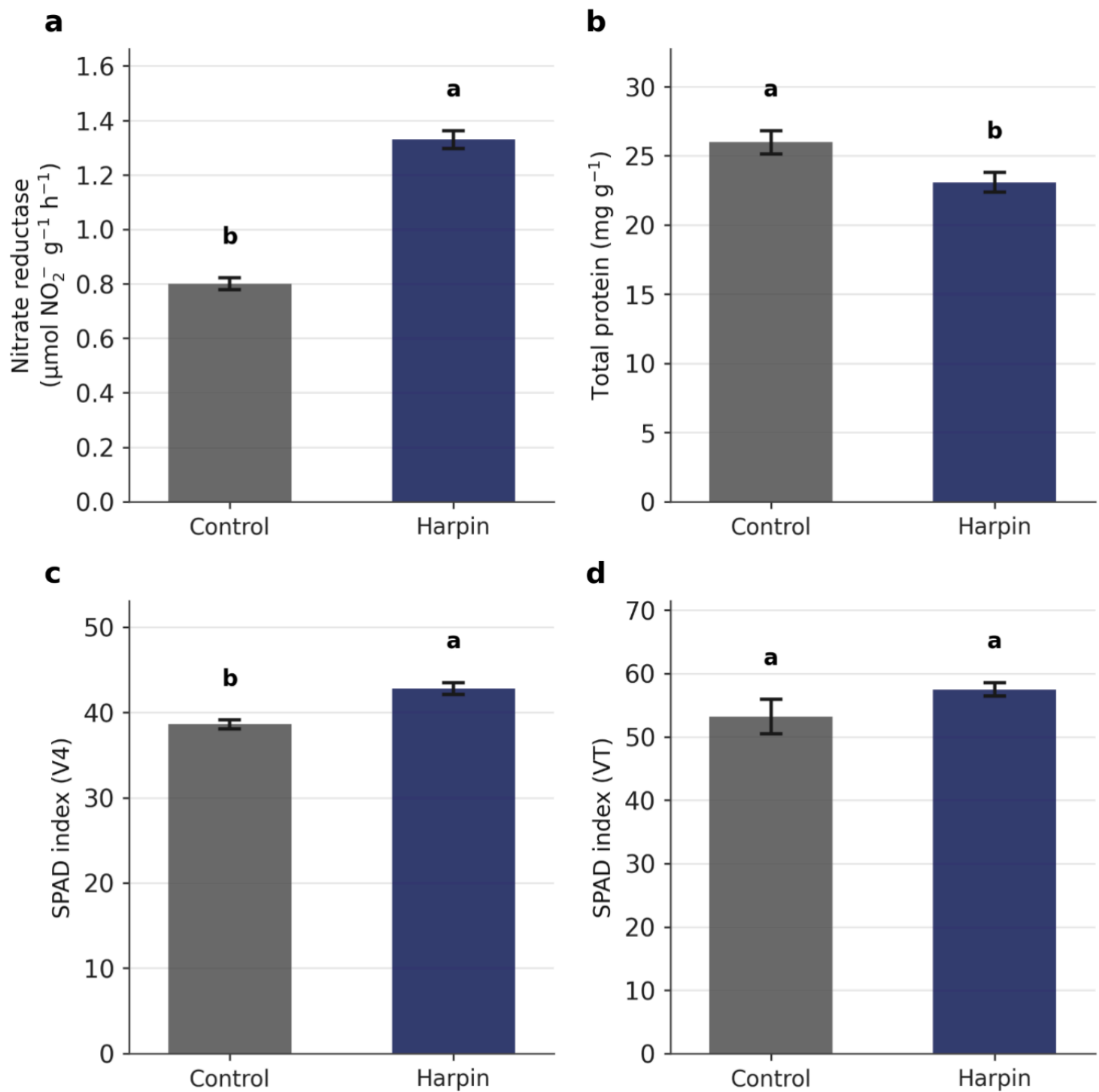


Figure 3

Effect of HrpNEA applied in seed treatment on: (a) activity nitrate reductase, (b) total protein content, (c) and (d) chlorophyll content (SPAD units) at V4 and VT growth stages respectively in maize hybrid DKB 390 PRO. Means followed by the same letter do not differ (randomized complete block ANOVA, $P \leq 0.05$, equivalent to a block-paired t-test; $n = 6$ blocks).

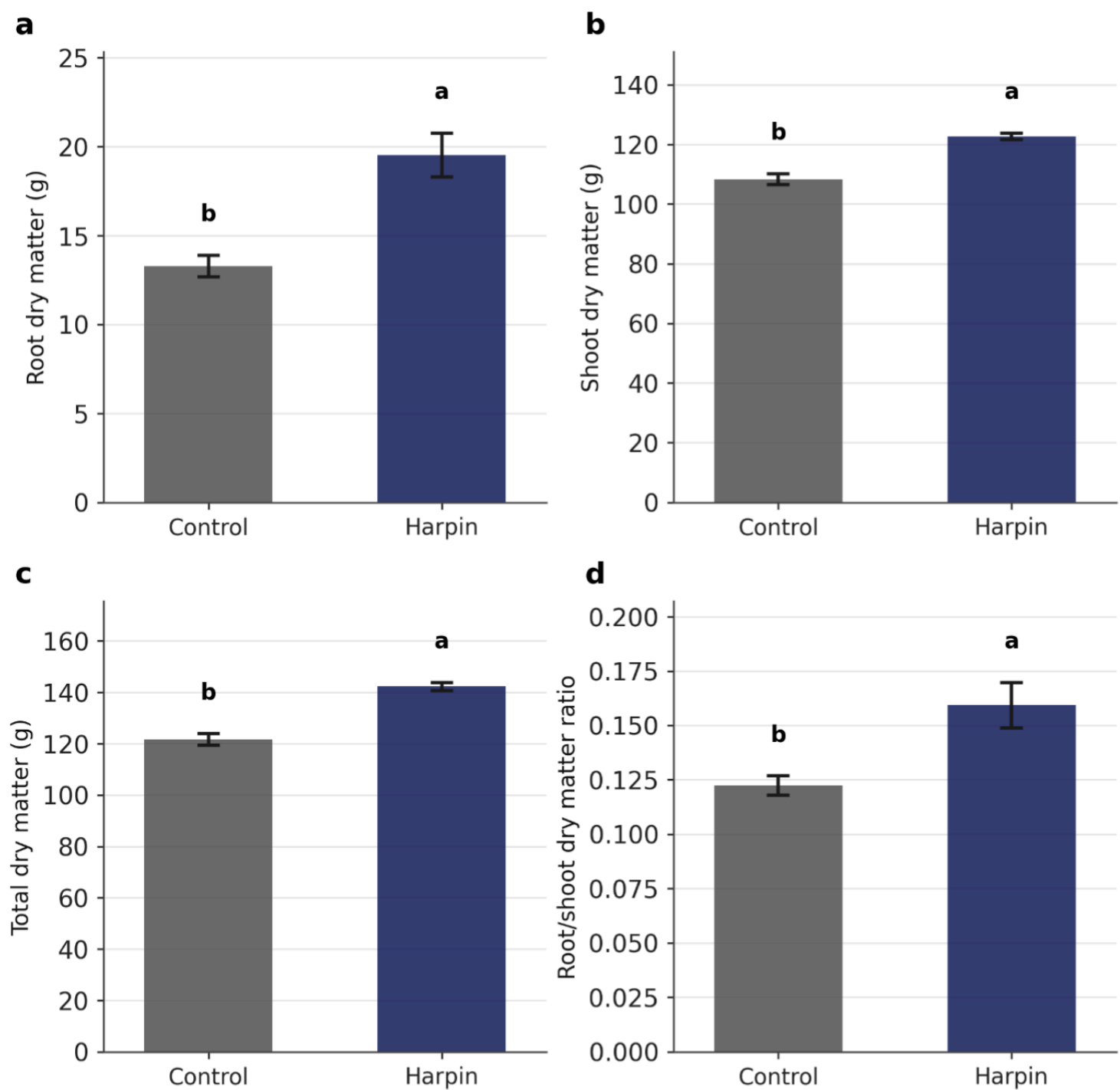


Figure 4

Effect of HrpNEA protein applied in seed treatment on: (a) root dry matter; (b) shoot dry matter; (c) total dry matter and (d) root/shoot dry matter in maize hybrid DKB 390 PRO. Means followed by the same

letter do not differ (randomized complete block ANOVA, $P \leq 0.05$, equivalent to a block-paired t-test; $n = 6$ blocks).

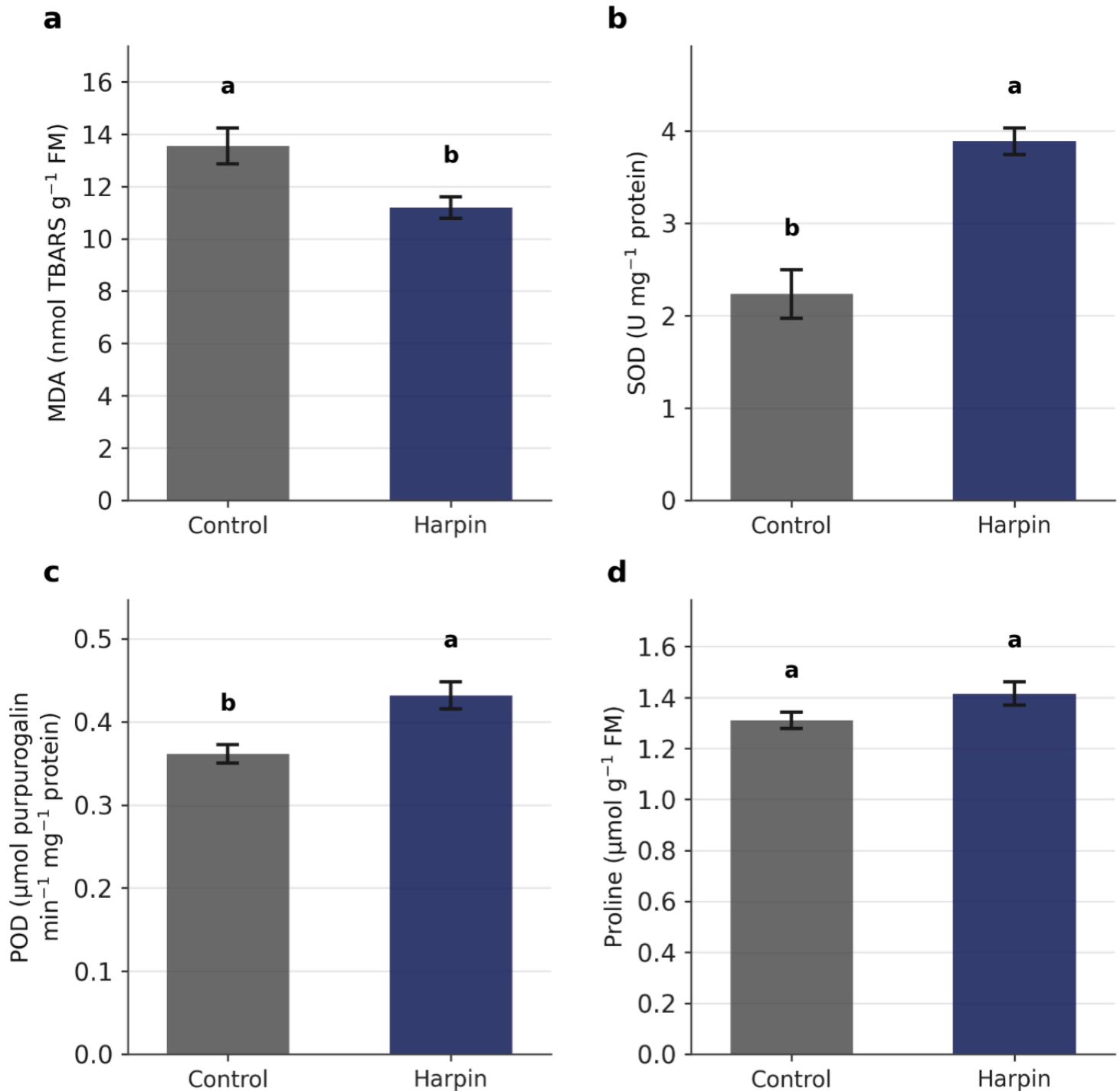


Figure 5

Effect of HrpNEA protein applied in seed treatment on: (a) malondialdehyde (MDA, nmol [TBARS] g^{-1} [FM]), (b) superoxide dismutase; (c) peroxidase and (d) proline (μ mol [proline] g^{-1} [FM]) in maize hybrid DKB 390 PRO. Means followed by the same letter do not differ (randomized complete block ANOVA, $P \leq 0.05$, equivalent to a block-paired t-test; $n = 6$ blocks).

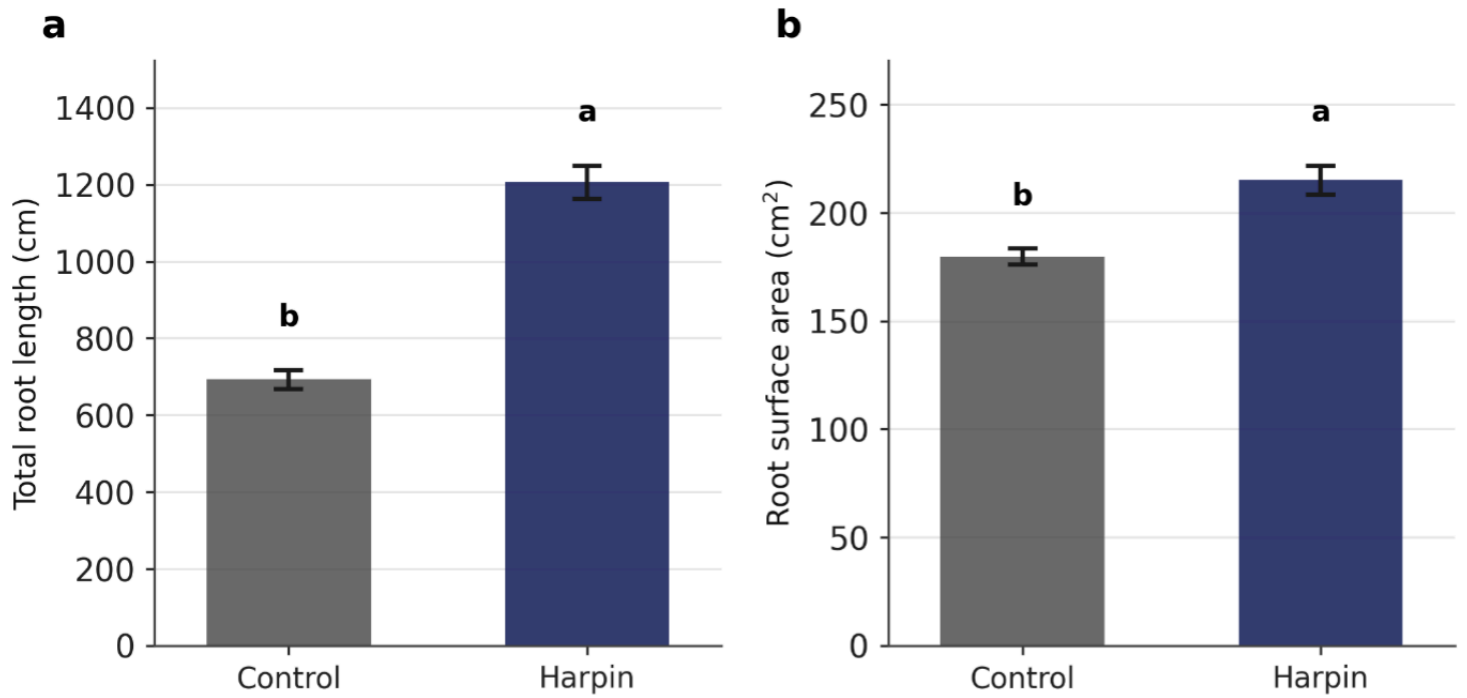


Figure 6

Effect of HrpNEA applied in seed treatment on: (a) total root length (cm) and (b) root surface area (cm²) in maize hybrid DKB 390 PRO. Means followed by the same letter do not differ (randomized complete block ANOVA, $P \leq 0.05$, equivalent to a block-paired t-test; $n = 6$ blocks).

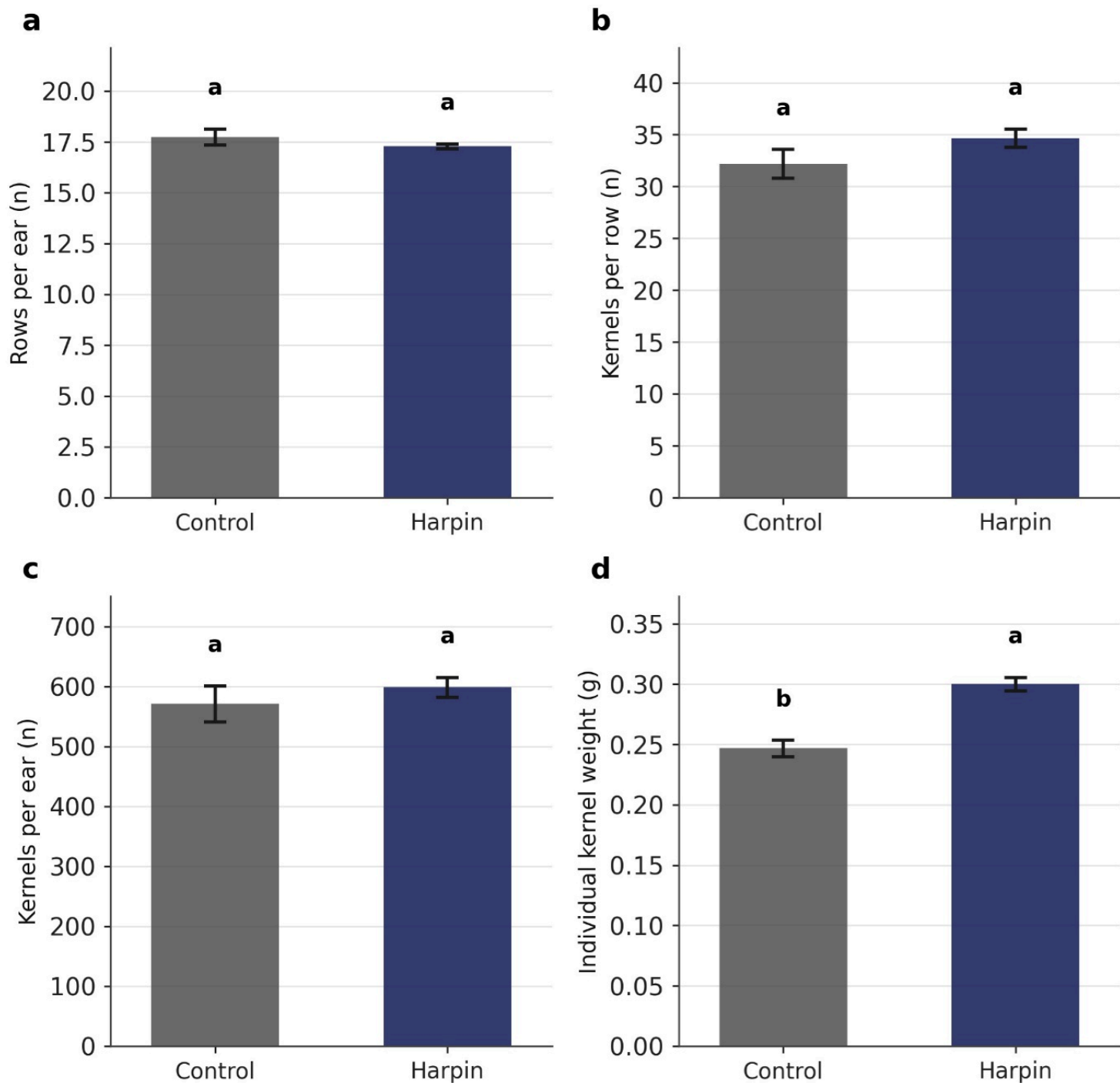


Figure 7

Effect of HrpNEA applied in seed treatment on number of rows per ear, number of kernels per row, number of kernels per ear, individual kernel weight in maize hybrid DKB 390 PRO. Means followed by the same letter do not differ (randomized complete block ANOVA, $P \leq 0.05$, equivalent to a block-paired t-test; $n = 6$ blocks).

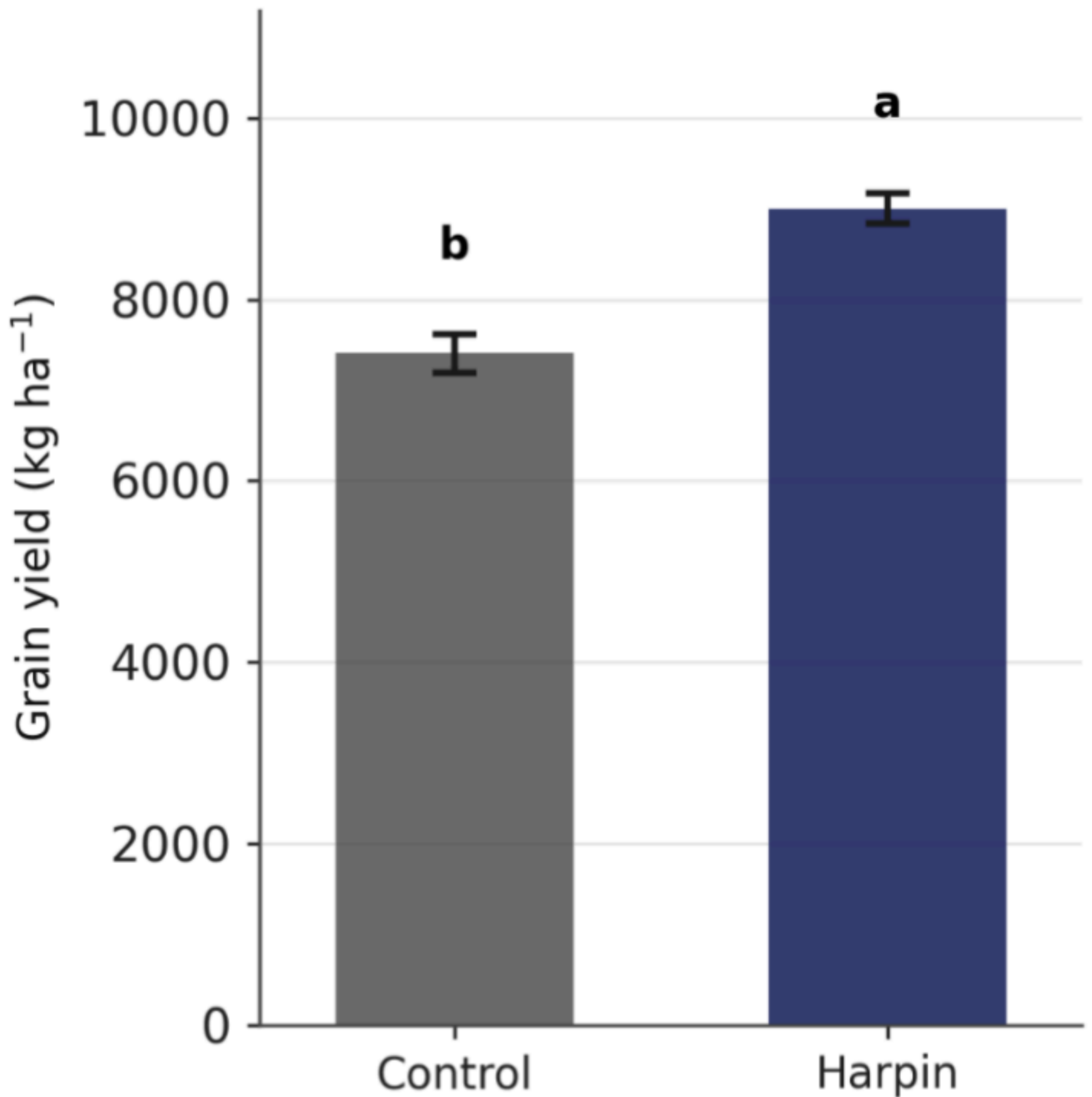


Figure 8

Effect of HrpNEA applied in seed treatment on grain yield in maize hybrid DKB 390 PRO. Means followed by the same letter do not differ (randomized complete block ANOVA, $P \leq 0.05$, equivalent to a block-paired t-test; $n = 6$ blocks).

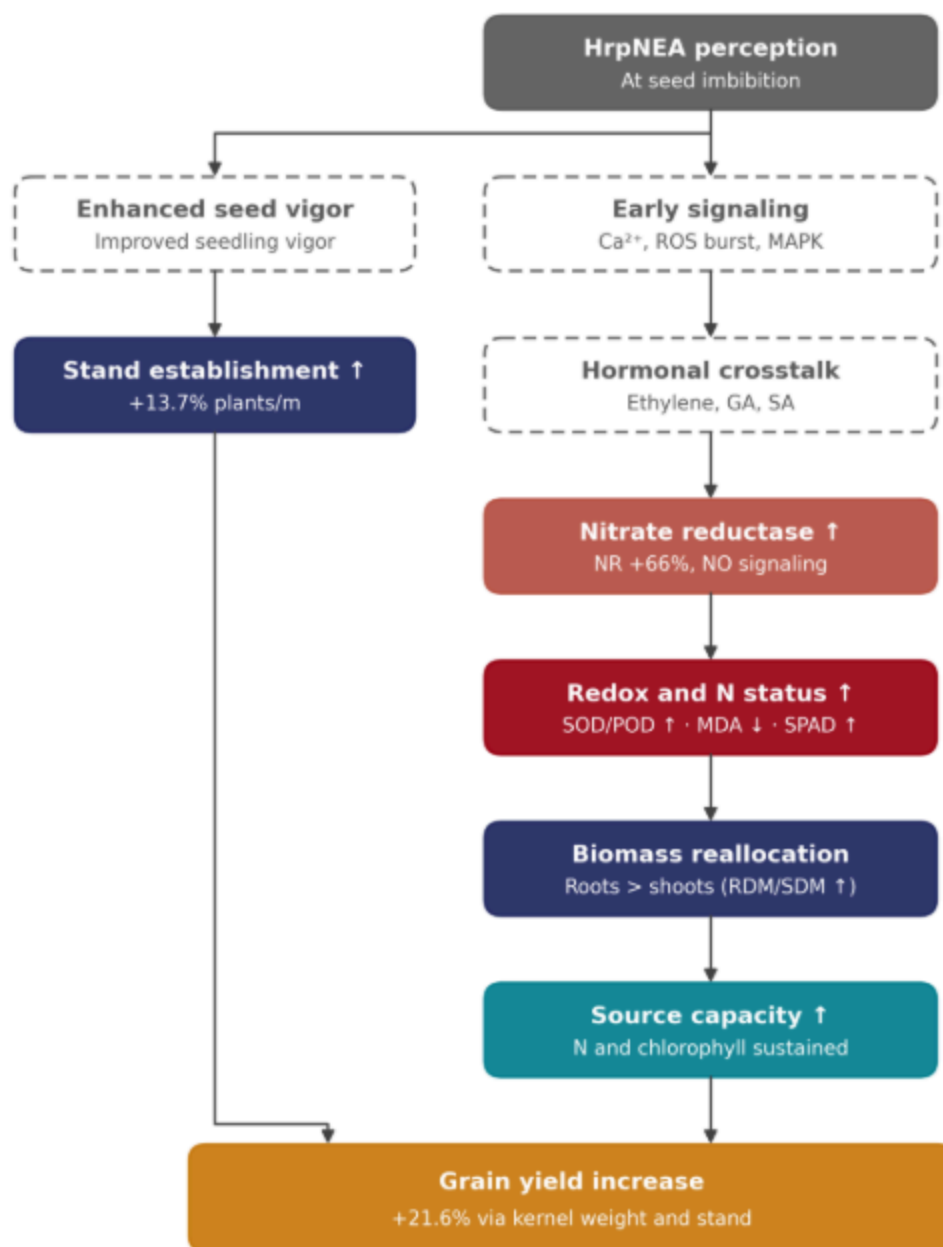


Figure 9

Proposed physiological mechanism linking HrpNEA seed treatment to stand establishment and grain yield in maize under water-limited field conditions. Solid-outlined boxes indicate traits measured in this study; dashed-outlined boxes indicate signaling steps inferred from the harpin elicitor literature but not directly assessed here.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterialHarpinCantaoJPGR.docx](#)