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Deciphering the morpho-physiological and biochemical reprogramming of fodder beet (Beta vulgaris L.) under variable drip irrigation and nitrogen fertigation in an arid ecosystem

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Abstract

Fodder beet (*Beta vulgaris* L.) is an emerging forage crop in arid regions, which requires precision input management to secure high yield. This study investigated the morpho-physiological and biochemical reprogramming of fodder beet (*Beta vulgaris* L.) under four drip irrigation levels (125%, 100%, 75%, and 50% IW/CPE) and two nitrogen doses (100% and 75% of 150 kg ha^{-1}) over 4 and 5 split fertigation schedules in the Thar Desert, India. Results revealed that severe water stress (50% IW/CPE) forced resource allocation trade-off and reduced leaf and root fresh weights by 27.5% and 29.1%, respectively. To mitigate oxidative and osmotic damage, the crop strategically boosted leaf antioxidant enzymes (catalase and peroxidase) while shifting its defense to the roots through the accumulation of osmolytes (proline, soluble sugars) and protective secondary metabolites (phenols, tannins). Reducing the N fertilizer dose by 25% reduced leaf and root biomass, as well as leaf chlorophyll, but PCA analysis revealed its neutral effect on drought-survival mechanisms. Multivariate principal component analysis showed that shifting to 5 splits subtly optimized canopy light-harvesting pigment configurations. These findings conclude that water management dictates primary survival in arid zones, while nitrogen splitting acts as a secondary, independent modifier of baseline pigment efficiency.

Introduction

Arid and semi-arid ecosystems span roughly 41% of the global landmass [1, 2] and are characterized by low & erratic rainfall, extreme temperatures, high potential evapotranspiration and limited water resources [3]. In India, hot arid regions comprise 31.7 million hectares, with the state of Rajasthan containing the largest fraction (61%) within the fragile Thar Desert landscape [4]. In these severe environmental conditions, animal husbandry forms the socioeconomic foundation of rural societies, contributing over 50% to the economy of arid districts of Rajasthan [5, 6]. Despite reliance on animal husbandry, less than 2% of the regional land area is dedicated to forage production due to competing preferences for commercial cash crops under limited water allocation. [7]. Consequently, acute edapho-climatic constraints result in persistent green fodder deficits, particularly during lean winter periods, forcing livestock dependency on low-quality crop residues, which degrades animal health and milk yields [8, 9].

Fodder beet (*Beta vulgaris* L.) has recently emerged as a highly promising forage crop to address the feed crisis due to its high biomass potential, outstanding nutritional value, high palatability, and remarkable tolerance to marginal and salt-affected soils [10, 11]. Despite this, sustaining forage yield in sandy soils under hyper-arid conditions is constrained by low water and nutrient availability [12]. Water deficit directly disrupts the soil-plant-atmosphere continuum, reduce plant leaf expansion, impairs photosynthetically active radiation (PAR) absorption, and cause the overproduction of reactive oxygen species (ROS) through electron transport chain disruption in chloroplasts and mitochondria [13–15]. To neutralize oxidative damage, plants trigger enzymatic or non-enzymatic antioxidant defense network and accumulate low-molecular-weight organic osmolytes to preserve cell turgor and membrane integrity [16]. Studies on *Beta vulgaris* L. have consistently shown that water stress leads to significant reductions in

growth attributes, relative water content, and membrane stability, while increasing osmolyte accumulation and antioxidant enzyme activity [17, 18]. The severity and duration of water deficit play a critical role in shaping these responses [19].

Concurrently, nitrogen (N) is a major crop production bottleneck in arid sandy soils, which are inherently deficient in soil organic matter and prone to severe leaching [20]. While N is essential for chlorophyll biosynthesis, rubisco activity and carbon assimilation, its root uptake relies heavily on adequate soil moisture to facilitate mass flow and diffusion pathways[21–23]. Water stress reduce transpiration pull and cell turgor, leads to reduction in the uptake and transport of nitrogen [24]. It also restricts nitrogen assimilation through the inhibition of enzymes involved in nitrogen metabolism, such as nitrate reductase and glutamine synthetase [25]. Conversely, high N application induced a reduction in mesophyll conductance, which limits photosynthetic capacity and N-use efficiency [26]. However, optimized N management can enhance root plasticity allowing plants to extract more of the water available from the soil [27]. Nitrogen increases leaf area index (LAI), providing ground cover that reduces soil surface evaporation and increases water use efficiency (WUE). This intricate relationship between nitrogen availability and water deficit emphasizes the importance of nitrogen management in mitigating the adverse impacts of water deficit [28].

While individual crop responses to water and nitrogen stress are documented globally, systematic field evaluations exploring the integrated effects of irrigation and nitrogen management (fertigation) on the physiological stability and biochemical defense mechanism of fodder beet in arid soils are scarce. To address this knowledge gap and decipher the interactive structural, physiological, and biochemical reprogramming of fodder beet within the hot arid agro-climatic conditions of Rajasthan, India, this study was executed with the following specific objectives:

1. Quantify the morpho-physiological threshold and biomass yield reductions of fodder beet under varying drip irrigation levels in hyper-arid sandy soils.
2. Characterize the tissue-specific biochemical reprogramming (leaves versus roots) regarding osmolyte accumulation, enzymatic antioxidant upregulation, and non-enzymatic secondary metabolite pathways under moisture and nutrient gradients to map individual organ defense behaviours.
3. Evaluate the efficiency of nitrogen application rates and splitting schedules under localised drip fertigation to determine their potential to mitigate water stress and enhance structural biomass.
4. Decipher the multi-variate structural and metabolic trade-offs using principal component profiling to establish optimised agronomic management strategies for maximising productivity and securing crop survival under severe resource constraints.

Results

The multi-factorial analysis of variance (ANOVA) indicated that irrigation water was the primary source of variance, which significantly ($p < 0.05$) influenced growth, physiological, and biochemical parameters.

The nitrogen doses (ND) significantly influenced biomass but remained unaffected for most physiological and biochemical parameters, except for enhanced levels of total chlorophyll in the leaf and total flavonoid content in the root. The nitrogen scheduling (NS showed no significant effect on any of the evaluated parameters. All two-and three-way interactions between IW, ND and NS for measured parameters were non-significant (Table 1).

Table 1
Analysis of variance for the effects of irrigation water (IW), nitrogen dose (ND) and nitrogen scheduling (NS) on fodder beet.

	Source of variation						
	IW	**ND**	**NS**	**IW × ND**	**IW × NS**	**ND × NS**	**IW × ND × NS**
df	3	1	1	3	3	1	3
Leaf fresh weight (q ha^{-1})	***	**	ns	ns	ns	ns	ns
Root fresh (q ha^{-1})	***	**	ns	ns	ns	ns	ns
Leaf dry weight (q ha^{-1})	***	*	ns	ns	ns	ns	ns
Root dry weight (q ha^{-1})	***	**	ns	ns	ns	ns	ns
Relative water content (%)	***	ns	ns	ns	ns	ns	ns
Membrane stability index (%)	***	ns	ns	ns	ns	ns	ns
Total chlorophyll (mg g^{-1} FW)	*	*	ns	ns	ns	ns	ns
Anthocyanin (mg g^{-1} FW)	ns	ns	ns	ns	ns	ns	ns
Proline in leaf (µg g^{-1} FW)	***	ns	ns	ns	ns	ns	ns
Proline in root (µg g^{-1} FW)	***	ns	ns	ns	ns	ns	ns
Total soluble sugars in leaf (%)	ns	ns	ns	ns	ns	ns	ns
Total soluble sugars in root (%)	***	ns	ns	ns	ns	ns	ns
Total soluble protein in leaf (mg g^{-1} FW)	***	ns	ns	ns	ns	ns	ns
Total soluble protein in root (mg g^{-1} FW)	***	ns	ns	ns	ns	ns	ns
Peroxidase activity in leaf (EU g^{-1} FW min^{-1})	***	ns	ns	ns	ns	ns	ns
Peroxidase activity in root (EU g^{-1} FW min^{-1})	***	ns	ns	ns	ns	ns	ns
Catalase activity in leaf (EU g^{-1} FW min^{-1})	***	ns	ns	ns	ns	ns	ns

'ns' indicates non-significant difference, while *, ** and *** denote significance levels at alpha 0.05, 0.01 and 0.001%, respectively.

	Source of variation						
	IW	ND	NS	IW × ND	IW × NS	ND × NS	IW × ND × NS
Catalase activity in root (EU g^{-1} FW min^{-1})	***	ns	ns	ns	ns	ns	ns
Antioxidant capacity in leaf (FRU g^{-1} FW)	ns	ns	ns	ns	ns	ns	ns
Antioxidant capacity in root (FRU g^{-1} FW)	ns	ns	ns	ns	ns	ns	ns
Total phenol in leaf (µg catechol eq. g^{-1} FW)	**	ns	ns	ns	ns	ns	ns
Total phenol in root (µg catechol eq. g^{-1} FW)	***	ns	ns	ns	ns	ns	ns
Total flavonoid in leaf (mg quercetin eq. g^{-1} FW)	ns	ns	ns	ns	ns	ns	ns
Total flavonoid in root (mg quercetin eq. g^{-1} FW)	***	***	ns	ns	ns	ns	ns
Total tannin in leaf (µg catechin eq. g^{-1} FW)	**	ns	ns	ns	ns	ns	ns
Total tannin in root (µg catechin eq. g^{-1} FW)	***	ns	ns	ns	ns	ns	ns
Saponins in leaf (mg diosgenin eq. g^{-1} FW)	***	ns	ns	ns	ns	ns	ns
Saponins in root (mg diosgenin eq. g^{-1} FW)	**	ns	ns	ns	ns	ns	ns

'ns' indicates non-significant difference, while *, ** and *** denote significance levels at alpha 0.05, 0.01 and 0.001%, respectively.

Crop Yield and Biomass Accumulation

Data in Table 2 revealed that elevated irrigation (125% IW/CPE) produced the highest leaf and root fresh weights at 90 days after sowing (411.5 ± 27.9 and 327.2 ± 24.8 q ha^{-1}, respectively), significantly outperforming all other irrigation levels. Biomass production reduced progressively with decreasing irrigation water. Severe water stress (50% IW/CPE) significantly reduced leaf and root fresh weights by 27.5% and 29.1%, and dry weights by 13.2% and 21.5%, respectively, compared to 100% IW/CPE. Reduced nitrogen applications (75% RDN) significantly lowered leaf and root fresh yields by 7.1% and

5.7%, respectively, and corresponding dry biomass yields, compared to the 100% RDN benchmark. NS showed no statistical difference between applying nitrogen in 4 splits or 5 splits for both leaf and root fresh and dry weights.

Table 2
Effect of irrigation water (IW), nitrogen dose (ND) and nitrogen scheduling (NS) on leaf and root fresh and dry weight of fodder beet.

Treatments	Leaf fresh weight ($q\ ha^{-1}$)	Root fresh weight ($q\ ha^{-1}$)	Leaf dry weight ($q\ ha^{-1}$)	Root dry weight ($q\ ha^{-1}$)
Irrigation water				
125% IW/CPE	411.5 ± 27.9a	327.2 ± 24.8a	23.5 ± 1.9a	36.2 ± 2.7a
100% IW/CPE	366.6 ± 29.2b	295.3 ± 23.1b	21.9 ± 1.8b	33.0 ± 2.6b
75% IW/CPE	289.2 ± 29.2c	234.4 ± 17.0c	19.3 ± 1.9c	29.1 ± 2.1c
50% IW/CPE	265.9 ± 28.0d	209.4 ± 15.8d	19.0 ± 2.1c	25.9 ± 1.2d
Nitrogen dose				
100% RDN	345.5 ± 72.6a	274.4 ± 56.2a	21.6 ± 2.9a	32.0 ± 5.0a
75% RDN	321.1 ± 55.9b	258.8 ± 46.0b	20.3 ± 2.3b	30.1 ± 3.8b
Nitrogen scheduling				
4 Splits	329.6 ± 66.9a	268.1 ± 52.5a	20.7 ± 2.7a	31.0 ± 4.6a
5 Splits	337.0 ± 64.9a	265.0 ± 51.3a	21.2 ± 2.6a	31.1 ± 4.4a

Different lowercase letters within each column indicate statistically significant differences among treatments ($p < 0.05$) based on LSD post-hoc testing. Data are presented as mean ± SD.

Cell Water Status and Cellular Membrane Integrity

Leaf Relative Water Content (RWC) and Membrane Stability Index (MSI) decreased progressively alongside increasing water stress (Fig. 1). The absolute values for RWC and MSI decreased by 10.76% and 10.1%, respectively when irrigation water decreased from 125% IW/CPE down to 50% IW/CPE. Alterations in nitrogen doses and split schedules yielded no statistically significant shifts in either RWC or MSI values.

Photosynthetic and Photoprotective Pigments

Higher leaf total chlorophyll (TChl) content was recorded under water stress conditions (Fig. 2). The 50% IW/CPE recorded the highest chlorophyll content in the leaf (0.554 mg g^{-1} FW), which was significantly

higher than the 125% IW/CPE treatment but statistically comparable to 100% IW/CPE. Conversely, lowering the nitrogen dose to 75% RDN significantly decreased leaf chlorophyll content by 7.18% (0.552 mg g^{-1} FW) compared to full N fertilization (100% RDN). The nitrogen scheduling did not show significant differences, though nitrogen application in 5 splits (0.538 mg g^{-1} FW) slightly increased chlorophyll content compared to 4 splits (0.529 mg g^{-1} FW). Although water deficit induced a prominent upward trend in leaf anthocyanin content, the variations across irrigation treatments were not statistically significant (Fig. 2).

Osmolyte Dynamics (Proline and TSS)

Fodder beet subjected to severe water stress (50% IW/CPE) showed significantly higher proline accumulation in both the leaf (13.3%) and root (41.9%) compared to 100% IW/CPE (Fig. 3). Under water stress, total soluble sugars showed a marginal increase in the leaves but increased significantly in the roots (Fig. 3).. Plants received 75% RDN accumulated lower proline in the leaf (121.3µg g^{-1} FW) than those that received 100% RDN (132.2µg g^{-1} FW), although there was no significant effect of N doses on root osmolytes. The NS did not significantly influence either proline or soluble sugar content.

Soluble Proteins and Antioxidant Enzymes

As anticipated, water stress significantly influenced TSP as well as activities of antioxidant enzymes such as POD and CAT in both leaf and root (Fig. 4). Severe water stress (50% IW/CPE) significantly increased total soluble protein (TSP) values, showing a 15.1% increase in leaves (20.34 to 23.41 mg g^{-1} FW) and a 13.0% increase in root systems (3.46 to 3.91 mg g^{-1} FW) over 100% IW/CPE. Enzymatic antioxidant markers (POD & CAT) demonstrated a strong response to water stress. Peroxidase (POD) activities rose by 42.9% in leaves and 37.6% in roots under 50% IW/CPE. CAT activity mirrored the same pattern, increased by 40.2% in leaves and 45.5% in roots under severe water stress (Fig. 4). In contrast, elevated irrigation (125% IW/CPE) significantly reduced leaf POD and CAT activities by 16.0% and 17.1%, respectively, without altering baseline root enzyme behaviours.

Total Antioxidant Capacity and Secondary Metabolites

Total Antioxidant Capacity (TAC) increased marginally under water stress, though these fluctuations lacked statistical significance across irrigation, nitrogen dose and split schedules (Fig. 5).

Water stress dramatically enhanced total phenolic content (TPC) across the plant (Fig. 5). Leaf phenol content climbed steadily from 397.00 µg g^{-1} FW with 125% IW/CPE to 442.28 µg g^{-1} FW with 50% IW/CPE. A similar trend was observed in the roots, where phenolic content increased by more than 73.1% from 78.51 µg g^{-1} FW to 135.88 µg g^{-1} FW under water stress. Total flavonoid content (TFC) in leaves remained stable under water stress, ranging from 31.02 to 33.86 mg g^{-1} FW (Fig. 6). While root

TFC increased significantly by 41.9% (from 0.86 mg g^{-1} FW at 125% IW/CPE to 1.22 mg g^{-1} FW under 50% IW/CPE) (Fig. 6). ND and NS did not significantly affect flavonoid content in leaves, whereas in the root, 100% RDN (1.21 mg g^{-1}) increased flavonoid content significantly by 22.2% over 75% RDN (0.99 mg g^{-1}). The nitrogen scheduling in 5 splits increased flavonoid content in the root. However, the difference was not statistically significant.

Total tannin (TT) values rose by 15.0% in leaves and 50.9% in roots under 50% IW/CPE treatments compared to 100% IW/CPE (Fig. 6). Saponin (SAP) content showed opposing trends between organs, where leaf saponin content decreased by 23.2% and root saponin concentrations increased significantly by 16.3% under severe water stress (Fig. 6).

Pearson Correlation Coefficients

The Pearson correlation analysis revealed significant relationships among morphological, physiological and biochemical traits in fodder beet plants under water stress (Fig. 7). Leaf fresh weight (LFW) and root fresh weight (RFW) exhibited an exceptionally strong positive correlation (r = 0.99), indicating synchronized growth between above and below-ground plant parts, and dry weights were also strongly correlated (r = 0.97), suggesting balanced biomass allocation. Relative water content (RWC) showed strong positive correlations with both LFW (r = 0.99) and RFW (r = 0.99), highlighting the critical role of maintaining turgor pressure on growth. The membrane stability index (MSI) was highly correlated with RWC (r = 0.96), reflecting their joint contribution to cellular integrity under stress conditions. Total chlorophyll and anthocyanin concentration in leaves were moderately associated (r = 0.65) and both showed positive correlation with leaf osmolytes, antioxidant enzymes, total flavonoids and tannin concentration in leaves.

Leaf and root FW and DW, RWC and MSI showed negative correlation with total chlorophyll, anthocyanin, osmolytes, antioxidant enzymes and secondary metabolites concentration in root and leaf, except for saponin concentration in leaf. Root proline and total soluble protein concentration showed strong negative correlation with leaf and root FW and DW, RWC and MSI (r = -0.80 to -0.99). Phenolic compounds, including total phenols and flavonoids, showed moderate to high positive correlation with antioxidant enzymes (r = 0.56 to 0.99), supporting their synergistic action in scavenging reactive oxygen species. Saponins in the leaf showed a negative correlation with other metabolites, except for Leaf and root FW and DW, RWC and MSI, where they showed a positive correlation.

Principal Component Analysis (PCA)

To understand how all the measured growth, physiological, and biochemical traits change together under different treatments, a Principal Component Analysis (PCA) was performed. The first two components explained 87.9% of the total variation in the study (PC1 explained 79.9% and PC2 explained 8.0%), showing that the analysis captures almost all the important data (Fig. 8). Statistical extraction of the

eigenvectors revealed that PCA 1 (eigenvalue: 22.38) was predominantly governed by hydraulic, morpho-structural, and cell integrity traits. The highest negative loading scores were contributed by RWC (-0.99), root fresh weight (-0.99), root dry weight (-0.98), leaf fresh weight (-0.97) and, whereas the highest positive loadings along this axis were driven by severe water stress indices, including leaf peroxidase activity (0.996), leaf total tannins (0.994) and root proline (0.98).

Conversely, PCA 2 (Eigen value: 2.25) was orthogonal to the moisture axis and was heavily loaded on its positive axis by traits sensitive to nitrogen scheduling and photoprotection, specifically leaf TAC (0.83), leaf anthocyanin accumulation (0.72), leaf flavonoids (0.39) and total chlorophyll (0.12). The Leaf TP (-0.48) and Leaf TSP (-0.47) were distributed in the negative direction of the axis along with 4 N splits.

Discussion

The present study provides a comprehensive elucidation of the individual and interactive effects of the irrigation water (IW) level, nitrogen dose (ND), and nitrogen scheduling (NS) on the growth, physiological performance, and biochemical reprogramming of fodder beet (*Beta vulgaris* L.).

In arid sandy soils, the soil-plant-atmosphere continuum dictates crop performance, where low soil moisture and high vapour pressure deficit reduce leaf turgidity, which can be measured by RWC [29, 30]. This loss of turgor induces cellular dehydration, which restricts cell expansion and size, prompts stomatal closure, and diminishes the photosynthetic rate, ultimately leads to reduced leaf area, suppressed growth, and lower biomass production [31]. Exposing fodder beet to severe water stress (50% IW/CPE) reduced leaf RWC by 7.9%, tripping a cascade that cut total biomass by nearly 30%. The strong positive correlation between RWC and fresh biomass ($r = 0.99$) confirms that tissue hydration is the primary driver of vegetative growth. Multivariate principal component analysis clearly maps this biological trade-off along the horizontal axis (PC1). Under optimum water availability (100% and 125% IW/CPE), treatments cluster near growth-associated vectors, indicating that stable moisture maintains the turgor pressure needed for cell division and structural expansion. Under severe stress, the data coordinates shift across the origin toward stress-responsive metabolic vectors. This mathematical shift shows that the plant actively halts vegetative growth to redirect its energy reserves into root osmo-protection and enzymatic detoxification (POD and CAT). Restricted stomatal conductance under water stress limits CO_2 fixation, generating excess excitation energy that triggers reactive oxygen species (ROS) and cellular damage [32, 33]. These radical species cause lipid peroxidation and raise membrane permeability, leading to electrolyte leakage [34, 35]. The strong correlation between RWC and MSI ($r = 0.96$) highlights their tight interdependence in preserving cellular turgor and membrane integrity [36]. To counter desiccation, fodder beet shifts fixed carbon away from canopy development to build protective solutes (osmolytes). Proline concentrations jumped by 13.3% in leaves and 41.9% in roots, while total soluble sugars rose significantly belowground (roots). Proline helps to lower osmotic potential, chelates metals, scavenges reactive oxygen species (ROS), protects the redox balance, reduces lipid peroxidation, and shields the photosynthetic apparatus [37] [38, 39]. Soluble sugars prevent protein denaturation during dehydration and act as energy reserves [40, 41]. The strong negative correlation ($r < -0.80$)

between these osmolytes and biomass confirms that this metabolic protection comes at the direct cost of structural growth [42].

Fodder beet mitigates oxidative stress through a synchronized enzymatic and non-enzymatic defense system. Leaf catalase and peroxidase activities increased by over 40% under severe stress. Peroxidase (POD) facilitates the oxidation of phenols to generate phenoxyl radicals ($PhO^{\bullet}$), commonly known as quinone acceptors. The strong negative correlations between these antioxidant enzymes and RWC ($r = -0.93$ to -0.98) confirm that their activation is a direct response to cellular water deficits. Total soluble protein is another indicator of antioxidant defense and osmoregulation, which involves amino acid metabolism, chaperones and structural proteins that maintain cellular integrity [43, 44]. The significant 15.1% rise in leaf and 13.0% rise in root of total soluble protein in fodder beet under water stress reflects increased synthesis of stress-responsive proteins [45].

The non-enzymatic secondary metabolites revealed a clear tissue-specific allocation strategy. Root total phenolics shot up by 79.7% and tannins by 51% under severe water stress. Flavonoids also showed tissue-specific responses, where root flavonoids increased 13% under water stress, and leaf flavonoids remained nearly stable. Saponins even displayed a linear re-translocation pattern, decreasing in leaves while increasing in roots. This preferential investment in belowground defense suggests a coordinated mechanism to protect root system integrity and maintain minimal water absorption under water stress [40, 46]. Phenolic compounds and flavonoids correlate moderately to strongly with CAT and POD ($r = 0.56$ to 0.99), highlighting their synergistic role in scavenging ROS.

Despite an overall reduction in biomass, water stress led to a 3.4% increase in chlorophyll content. While drought stress typically degrades chlorophyll, this increase points to a stress-induced compensatory mechanism sometimes observed after prolonged water stress [47, 48]. This recovery is supported by a simultaneous rise in photoprotective anthocyanins. The positive correlation between chlorophyll and anthocyanins ($r = 0.65$) and their shared alignment on the PCA biplot demonstrate that anthocyanins help shield the light-harvesting complex when stomata are closed under water stress.

While nitrogen availability is a major driver of agricultural productivity, lowering the nitrogen dose to 75% RDN only reduced biomass and pigment content without triggering core stress-defense pathways. In traditional surface irrigation systems across sandy desert soils, heavy and infrequent nitrogen applications are highly susceptible to leaching, where larger volumes typically expand adaptive variances. Under drip fertigation, the nutrients are delivered directly into the active root zone, stabilizing soil nitrogen concentrations and maximizing nutrient uptake efficiency. Total nitrogen doses clustered tightly at the zero-variance origin of the PCA biplot, proving that reducing the nitrogen dose by 25% has neutral effect on the metabolic drought-survival mechanisms of plant [49, 50].

In plant systems biology, individual physiological traits often display minor, non-significant fluctuations when analyzed in isolation due to micro-environmental canopy noise. Multivariate PCA overcomes this limitation by pooling the shared variance of related variables, effectively filtering out random background noise [51, 52]. In our research, although standalone ANOVA showed no isolated differences between

nitrogen split schedules, multivariate PCA unmasked a highly coordinated restructuring of the light-harvesting apparatus along the vertical axis (PC2). Shifting from 4 to 5 splits under drip fertigation aligns closely with total chlorophyll, anthocyanin, and total antioxidant vectors, indicates that increasing the fertigation schedule from 4 to 5 splits under the drip system into the active root zone stabilizes nitrogen availability, minimizing leaching in sandy soils and fine-tuning canopy light-use efficiency without showing dramatic jump in any single pigment concentration.

Because the pigment-modulating axis (PC2) runs entirely perpendicular to the high-variance moisture axis (PC1), it confirms that nitrogen management and water adaptation operate through independent biological pathways under water stress. When fodder beet enters survival mode under moisture stress, forcing growth through high fertilizer rates is metabolically ineffective. Therefore, field management in vulnerable arid landscapes must prioritize drip irrigation scheduling over high nitrogen rates.

Conclusion

This study reveals that water availability operates as the primary environmental factor for fodder beet (Beta vulgaris L.) in arid ecosystems, driving a distinct metabolic trade-off where the crop sacrifices vegetative biomass (~ 27–29% reduction) to invest in a highly coordinated, tissue-specific survival network. Under severe deficits (50% IW/CPE), leaves preferentially upregulate enzymatic antioxidant systems (CAT and POD) to preserve membrane stability, whereas roots accumulate osmo-protective proline, soluble sugars, and defensive secondary metabolites. Combined univariate and multivariate profiling proved that moisture levels and nutrient applications regulate separate biological systems. Total nitrogen doses cluster tightly at the zero-variance origin, demonstrating a neutral effect on core drought-survival pathways. Conversely, multivariate analysis unmasked a hidden pattern in nitrogen split scheduling that single-variable ANOVA missed, showing that fertigation scheduling coordinates subtle, collective adjustments in light-harvesting pigments along an orthogonal axis. Because these adaptive systems function in parallel, all factorial interactions remain statistically flat. Agronomically, trying to mitigate water stress through increased fertilizer volume is ineffective. Farmers in the Thar Desert should prioritize 125% IW/CPE drip irrigation regime coupled with conservative 75% recommended nitrogen dose applied over 5 split intervals to maximize resource-use efficiency and sustainably secure forage biomass yields.

Methods

Experimental Design and Crop Management

Fodder beet seeds of the Geronimo variety were sown in the second week of November at the research farm of ICAR-Central Arid Zone Research Institute, Jodhpur, India (26.2626° N, 72.9971° E). The experiment employed a factorial randomized block design (FRBD) with three replications. The treatments comprised four irrigation water (IW) levels (125% IW/CPE, 100% IW/CPE, 75% IW/CPE and 50% IW/CPE), two nitrogen doses (ND) (100% and 75% of recommended dose of nitrogen *i.e.*, 150 kg

ha^{-1}) and two nitrogen scheduling (NS) strategies: 25% basal nitrogen with 75% nitrogen applied *via* fertigation in three splits and 25% basal nitrogen with 75% nitrogen fertigated in four splits.

The crop was cultivated on raised beds in paired-row, with rows spaced 60 cm apart and plants within each row spaced 20 cm apart. Irrigation was provided through a drip system with an emitter discharge rate of 2 L/h, activated when cumulative pan evaporation (CPE) reached nearly 20 mm, as measured daily with a USWB Class A pan evaporimeter. The volume of water applied to each irrigation corresponded to the 20 mm CPE according to the treatments. The recommended dose of nitrogen (RDN) for fodder beet in arid Rajasthan is 150 kg ha^{-1}. The basal nitrogen dose was applied just before sowing, and fertigation was carried out between 30 and 60 days after sowing (DAS) at regular intervals, with nitrogen splits according to treatment specifications. At 90 DAS, plant samples were collected, flash-frozen in liquid nitrogen, and stored for subsequent biochemical analyses.

Plant Fresh Weight (FW) and Dry Weight (DW)

The plants from the two rows of 5.2 m were taken from each plot on the 90th day after sowing, and their fresh weights were measured using an electronic weighing balance. Subsequently, the plant samples were dried in a hot air oven at 60°C for 48 h and weighed to determine the dry weight (DW).

Leaf Relative Water Content (RWC)

Leaves from five plants per treatment were cut into disc shapes and immediately weighed to measure the fresh weight (FW). Subsequently, the leaves were immersed in deionized water and reweighed after 24 h to determine the turgid weight (TW). The leaves were then placed in a hot air oven at 60°C for 48 h to determine the dry weight (DW). The following formula was used to calculate the relative water content (%) [53]:

$$RWC\ (\%) = \left[(\mathrm{FW} - \mathrm{DW}) / (TW - DW) \right] \times\ 100$$

Membrane Stability Index (MSI)

The membrane stability index was determined according to the methodology outlined by [54]. Fresh leaf tissue (1 g) was cut into uniform discs and immersed in 10 mL of double-distilled water in test tubes, replicated twice. One set was incubated in a water bath at 40°C for 30 min, and the initial electrolyte leakage (C_1) was quantified using a calibrated conductivity meter. The second set was subjected to 100°C for 15 min to induce complete membrane disruption, and the maximum electrolyte leakage (C_2) was recorded. MSI was calculated using the following formula:

$$MSI = \left[1 - \left(C_1/C_2\right)\right] \times\ \ 100$$

Total Chlorophyll (TChl) and Anthocyanin (Antho)

For photosynthetic pigments quantification, 1 g of fresh leaf tissue was cut into small pieces and extracted in 10 mL of dimethyl sulfoxide (DMSO) under dark conditions until complete tissue decolorization was achieved. The resulting extract was filtered through Whatman filter paper, and the absorbance was recorded at 537, 647, and 663 nm using a UV spectrophotometer with DMSO serving as the blank. Total chlorophyll content was calculated using the equations of [55] and anthocyanin content was determined using the formula of [56] and expressed as mg g^{-1} fresh weight:

$$TChl = (7.15 \times \ A_{663}) + (18.71 \ \times \ A_{647})$$

$$Antho = \left(81.73 \ \times \ A\square\square\square\right) - \left(6.97 \ \times \ A\square\square\square\right) - (2.228 \ \times \ A\square\square\square)$$

Proline (PRO)

The proline concentration was measured using the method described by [57]. Briefly, fresh plant tissue (1 g) was homogenized in 10 mL of 3% (w/v) sulfosalicylic acid and centrifuged at 6000 rpm for 15 min at 4°C. The supernatant (2 mL) was reacted with an equal volume (2 mL) of each acid-ninhydrin and glacial acetic acid. This mixture was incubated at 100°C for 60 min in water bath and the reaction was terminated by rapid cooling in ice bath. The chromophore layer was extracted with 4 mL of toluene by vortexing and cooling to room temperature. The absorbance was measured at 520 nm using a UV-Vis spectrophotometer. The proline concentration was determined by interpolation from standard curve generated using known concentrations of L-proline and expressed as µg g^{-1} FW.

Total Soluble Sugars (TSS)

Total soluble sugar content was measured using the anthrone method. The 1 g plant sample was homogenized in 10 mL of ethanol (80%), centrifuged at 6000 rpm for 15 min, and the supernatant was stored in a refrigerator at 4°C for further analysis. Briefly, 100 µL of leaf aliquot and 10 µL of root aliquot were mixed with 4 mL of anthrone reagent (200 mg of anthrone dissolved in 100 mL of 95% ice-cold sulphuric acid). The mixture was heated for 8 min in boiling water bath and then rapidly cooled. Total soluble sugars were estimated using spectrophotometer at 630 nm, with glucose as the standard and the results were expressed as percentage on fresh weight basis.

Total Soluble Protein (TSP) and Antioxidant Enzyme Activities

Fresh leaf and root samples were collected, immediately flash-frozen in liquid nitrogen to halt metabolic activity and stored at -80°C until analysis. For enzyme extraction, 1 g of tissue was homogenized in 10

mL of 50 mM sodium phosphate buffer (pH 7.8) using pre-chilled mortar and pestle. The homogenate was centrifuged at 6000 rpm for 15 min at 4℃ and the resulting supernatant was stored at 4°C for subsequent biochemical assays.

Total soluble protein was estimated using the Lowry method. For leaf samples, 200 µL and for root samples, 500 µL aliquots were diluted to 1 mL with distilled water and a blank was prepared with 1 mL of distilled water. Freshly prepared 5 mL alkaline copper solution (mix 2% Na_2CO_3 in 0.1N NaOH and 0.5% copper sulphate in 1% sodium potassium tartrate in 50:1 ratio) was added to aliquot, mixed well and incubated for 10 mins at room temp. Afterwards, 0.5 mL of 1 N Folin-Ciocalteu reagent was added to the reaction mixture, mixed well and incubated in the dark at room temperature for 30 mins. The absorbance was measured at 660 nm against a blank. The amount of total soluble protein was calculated using bovine serum as standard and expressed as mg g^{-1} FW.

The reaction mixture for POD (5.2 mL) contained 2 mL of enzyme extract, 200 µL H_2O_2 (0.02%, v/v), 1 mL pyrogallol (0.1 N) and 2 mL of 0.1 M potassium phosphate buffer (pH 6.0). After thorough vortexing, the mixture was incubated at 37°C for 10 min. The absorbance was recorded at 430 nm using a spectrophotometer, with blank consisting of 2 mL enzyme extract and 3.2 mL phosphate buffer. Enzyme activity was expressed in enzyme units per gram of fresh weight per minute (EU g^{-1} FW min^{-1}) and calculated as follows:

$$Enzyme\ activity\ (EU\ g^{-1}\ FW\ min^{-1}) = 50\ \times\ \ Abs$$

The reaction mixture for CAT consisted of 500 µL of enzyme extract, 1 mL of H_2O_2 (200 mM), and 3.5 mL of potassium phosphate buffer (pH 7.0), which was kept at 37℃ for 3 min. For the assay, 2 mL of the reaction mixture was taken along with 2 mL potassium dichromate acetic acid (5% $K_2Cr_2O_7$ + glacial acetic acid in 1:3 ratio). The absorbance was measured at 570 nm against a blank. CAT activity was expressed in EU g^{-1} FW min^{-1} and calculated using the following formula:

$$Enzyme\ activity\ (EU\ g^{-1}\ FW\ min^{-1}) = 500\ \times\ \ Abs$$

Total Antioxidant Capacity (TAC) and Total Phenol (TP)

The 1 g plant sample was dissolved in 10 mL of 80% ethanol and sonicated at 35℃ for 60 min. The extracts were then centrifuged at 6000 rpm for 15 min. The supernatant was collected and stored at 4°C for further analysis.

The antioxidant capacity of sugar beet leaf and root extracts was assessed using the Ferric Reducing Antioxidant Power (FRAP) method, as outlined by [58]. The fresh FRAP reagent was prepared by mixing 20 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) and 20 mM $FeCl_3$ in ratio of 10:1:1. Then, 100 µL of the sample and 900 µL of water were mixed, followed by the addition of 3 mL FRAP reagent. The reaction was incubated at 37°C for 10 min, and the absorbance was measured at 593

nm using UV-vis spectrophotometer. The scavenging capacity of the samples was calculated using the formula and the results were expressed as FRU g^{-1} FW.

$$Antioxidant\ capacity\ (FRU\ g^{-1}\ FW) = 169.5\ \times\ \ Abs$$

The total phenolic content of the sample was determined using the Folin-Ciocalteu method. The collected supernatant was allowed to evaporate in open beakers at room temperature. After drying, the residue was reconstituted in 5 mL of distilled water. A 3 mL reaction mixture was prepared by mixing the sample aliquot with distilled water. Subsequently, 0.5 mL of Folin-Ciocalteu reagent was added, followed by 2 mL of 20% Na_2CO_3 solution after 3 min, which was vortexed and incubated in a water bath for one minute. A standard curve was created using catechol and the absorbance was measured at 650 nm against the reagent blank. The total phenol content was expressed as µg catechol eq. g^{-1} FW.

Total Flavonoid (TF), Total Tannins (TT) and Saponins (SAP) content

The plant samples were homogenized in 80% (v/v) methanol and centrifuged at 6000 rpm for 15 min. The supernatant was collected and stored at 4°C for further analysis.

The total flavonoid content was determined using modified colorimetric assay. Briefly, 500 µL of the extract was combined with 1.5 mL of 95% ethanol, 100 µL of 10% $AlCl_3$ and 100 µL of 1 M potassium acetate. The volume was adjusted to 5 mL with distilled water and the mixture was vortexed thoroughly. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm using UV-vis spectrophotometer. The TFC was quantified using quercetin standard curve and expressed as mg quercetin eq. g^{-1} FW.

The total tannin content was determined spectrophotometrically using the vanillin-HCl assay. The 1 mL sample extract was mixed with 5 mL of freshly prepared vanillin-HCl reagent (4% w/v vanillin and 8% v/v HCl in 1:1 ratio). The mixture was vortexed and incubated at room temperature for 20 min before use. The absorbance was measured at 500 nm using UV-vis spectrophotometer. The total tannin concentration was quantified against the catechin standard curve and expressed as µg catechin eq. g^{-1} FW.

For saponin content, 250 µL of leaf and 100 µL of root aliquots were taken and mixed with 250 µL vanillin HCl reagent and 2.5 mL of 72% H_2SO_4. The reaction mixture was kept in water bath at 60°C for 10 min, followed by cooling with ice-cold water. The absorbance was taken at 544 nm with a UV-vis spectrophotometer, and saponin content was measured against the diosgenin standard curve and expressed as mg diosgenin eq. g^{-1} FW.

Statistical Analysis

All experimental data are presented as mean ± standard deviation (SD) in tables and mean ± standard error (SE) in graphical representations, with three replicates. All graphs were prepared using R Studio (version 2025.09.2 + 418). For mean comparisons across treatments, the least significant difference (LSD) test was applied at the 5% probability level to determine statistical significance.

Declarations

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Author Contributions

K. conducted the field experiment, analyzed the data, and wrote the main manuscript text. S.P.S. supervised the experiment, assisted with manuscript writing, and provided necessary resources. M.K. provided guidance and support for laboratory analysis, fieldwork, and manuscript writing. M.P. provided guidance for the field experiment. M.S. assisted with laboratory work. M.K. guided the field experiment. A.S. assisted with fieldwork. P.K. guided the fieldwork and manuscript writing. All authors reviewed and approved the final manuscript.

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Additional Information

Competing Interests statement

The authors declare no competing interests.

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Figures

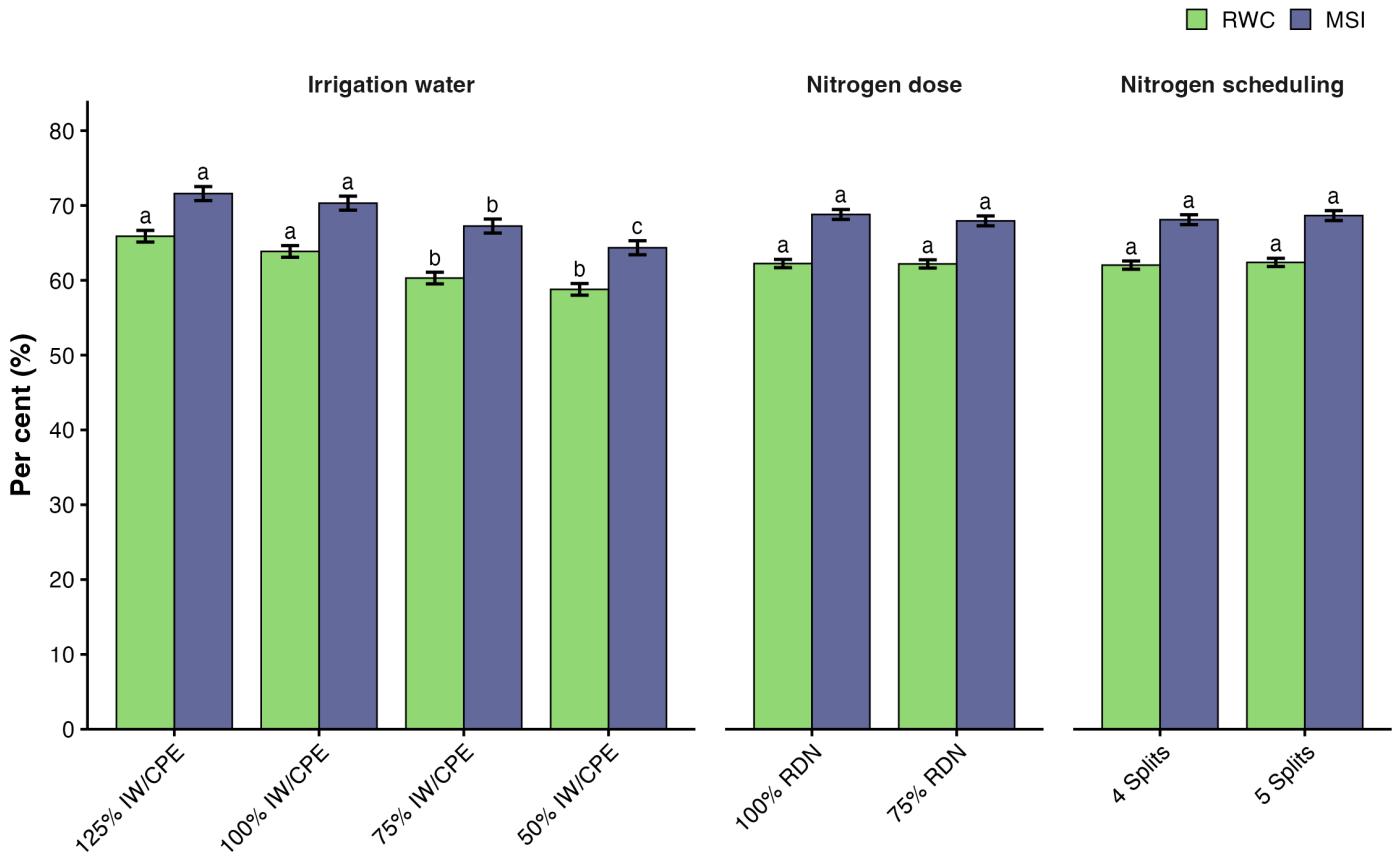

Figure 1

Effect of irrigation water, nitrogen dose and nitrogen scheduling on RWC (%) and MSI (%) in fodder beet leaf under drip irrigation.

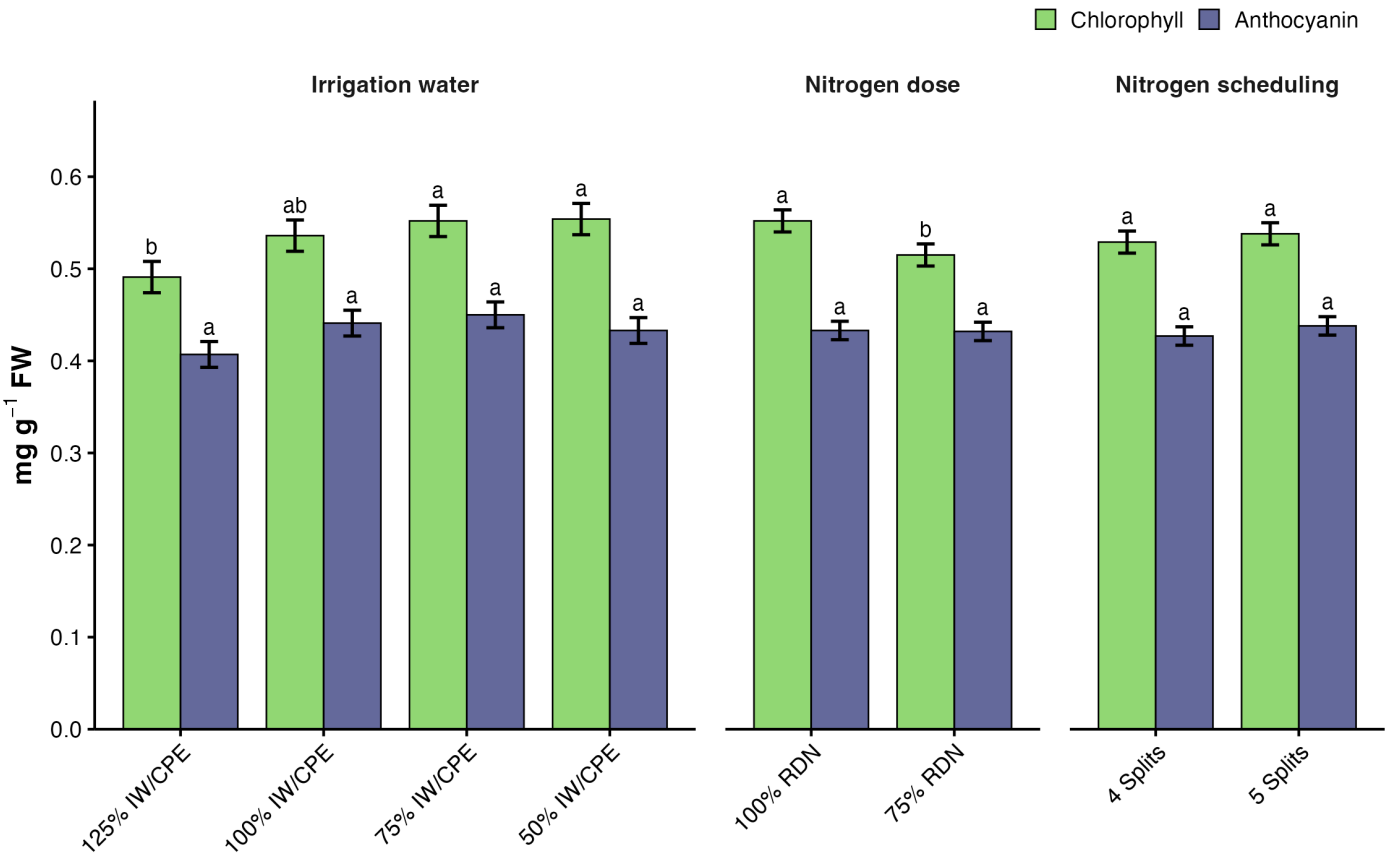

Figure 2

Effect of irrigation water, nitrogen dose and nitrogen scheduling on total chlorophyll (mg g^{-1} FW) and anthocyanin (mg g^{-1} FW) in fodder beet leaf under drip irrigation.

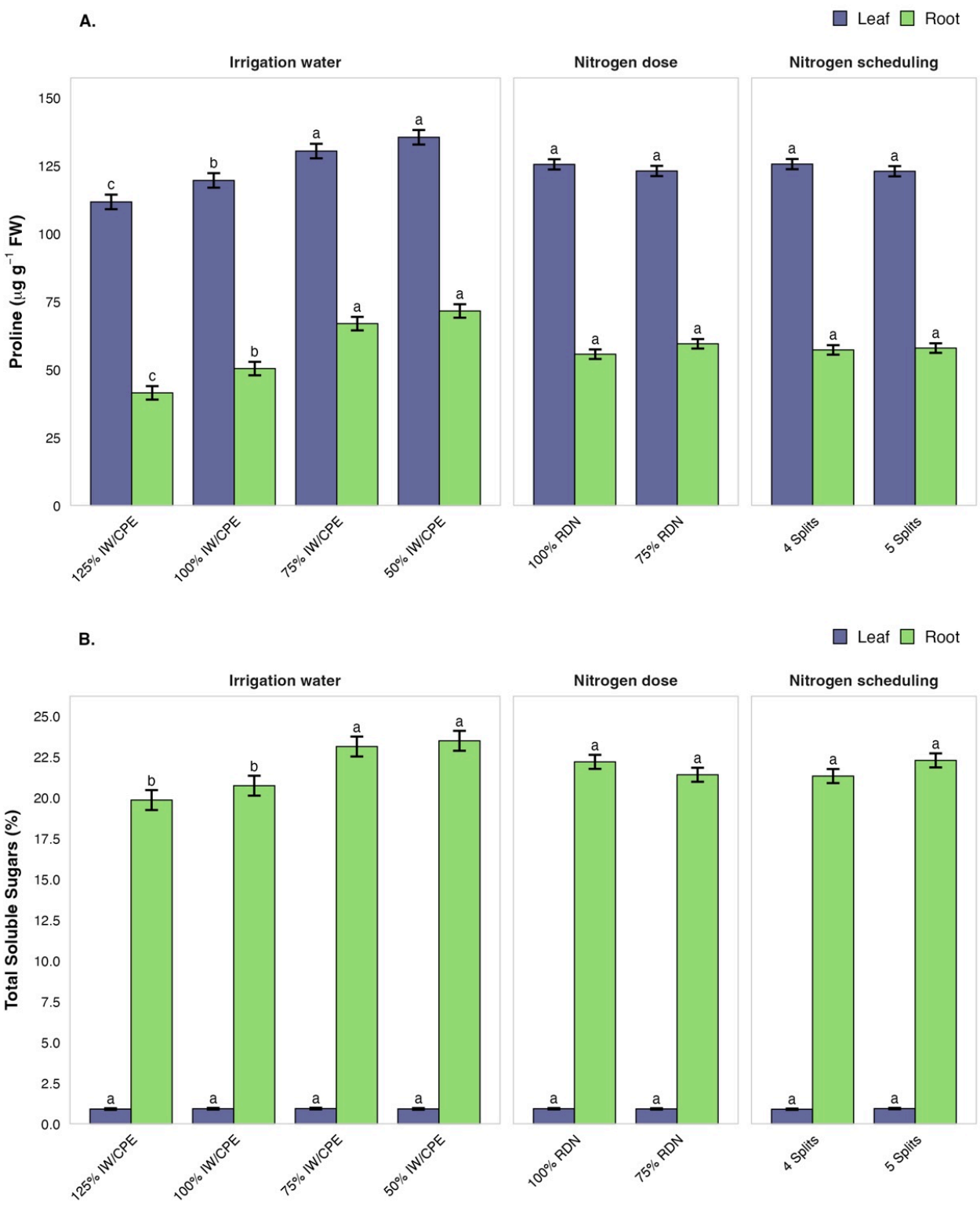

Figure 3

Illustrating the effect of irrigation levels, nitrogen dose and nitrogen scheduling on proline content (µg g^{-1} FW) and total soluble sugars (%) in fodder beet leaf and root under drip irrigation.

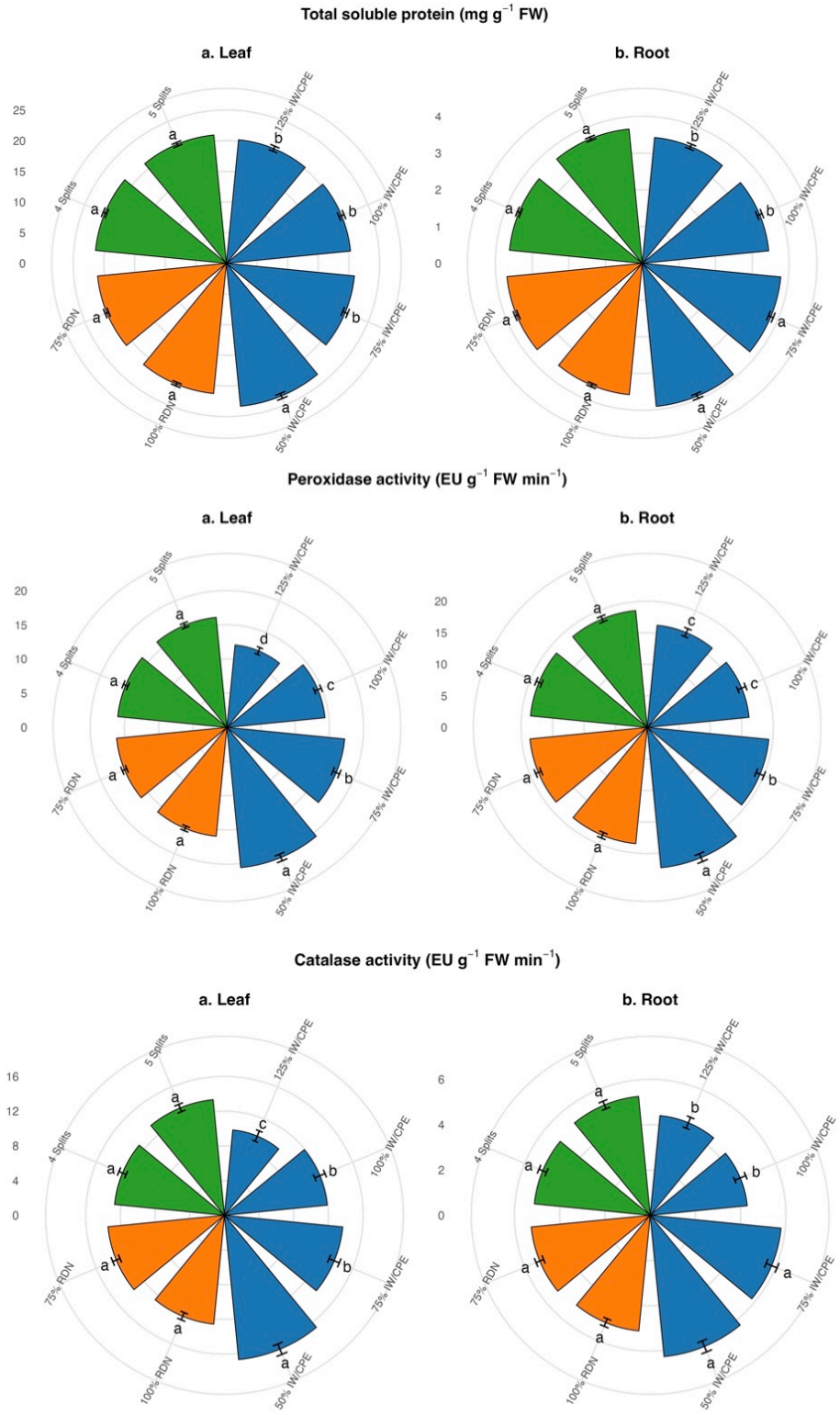

Figure 4

Effect of irrigation levels, nitrogen dose and nitrogen scheduling on total soluble protein (mg g^{-1} FW), peroxidase (EU g^{-1} FW min^{-1}) and catalase (EU g^{-1} FW min^{-1}) activity in fodder beet leaf and root under drip irrigation.

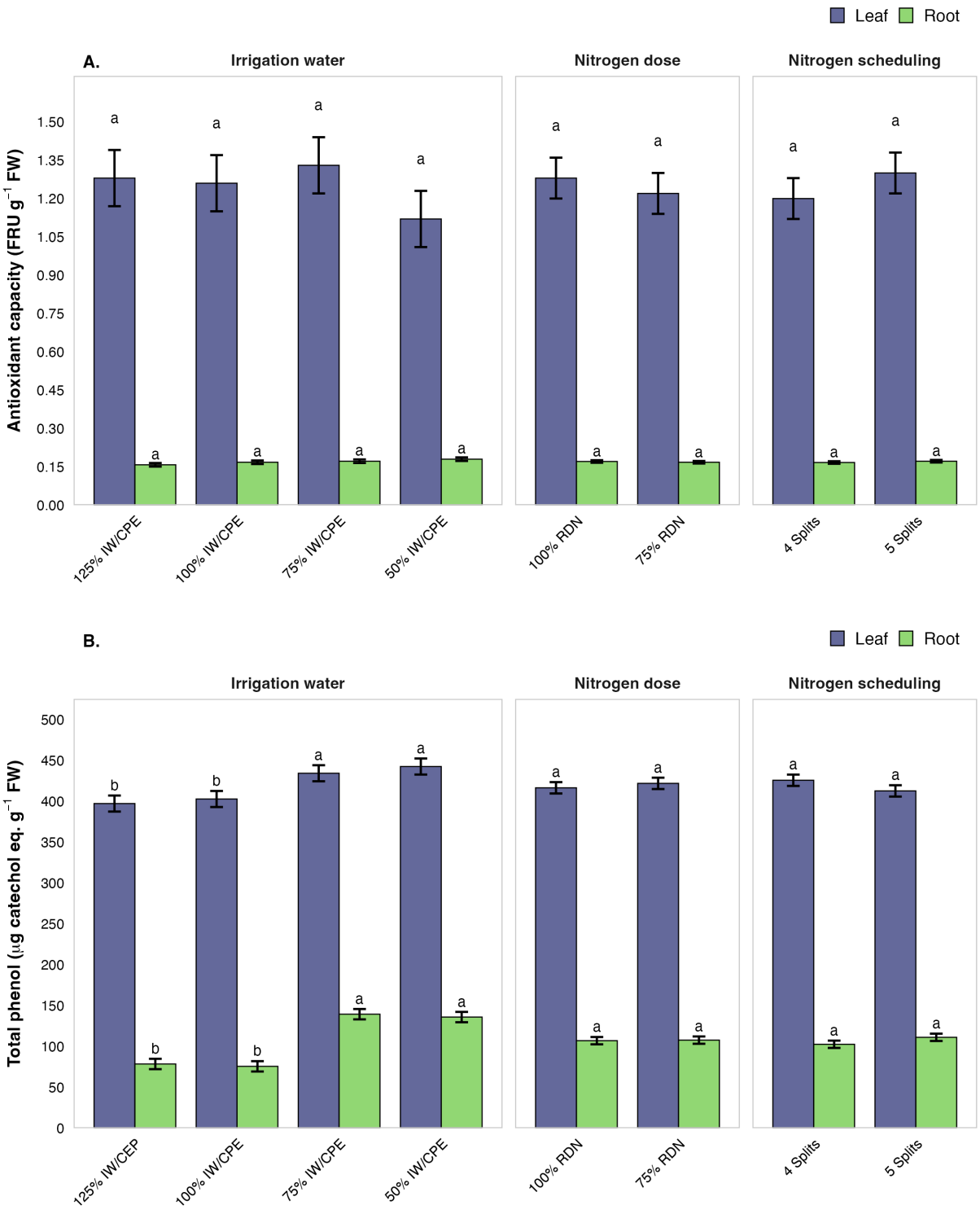

Figure 5

Effect of irrigation levels, nitrogen dose and nitrogen scheduling on total antioxidant capacity (FRU g^{-1} FW) and total phenols (µg catechol eq. g^{-1} FW) in fodder beet leaf and root under drip irrigation.

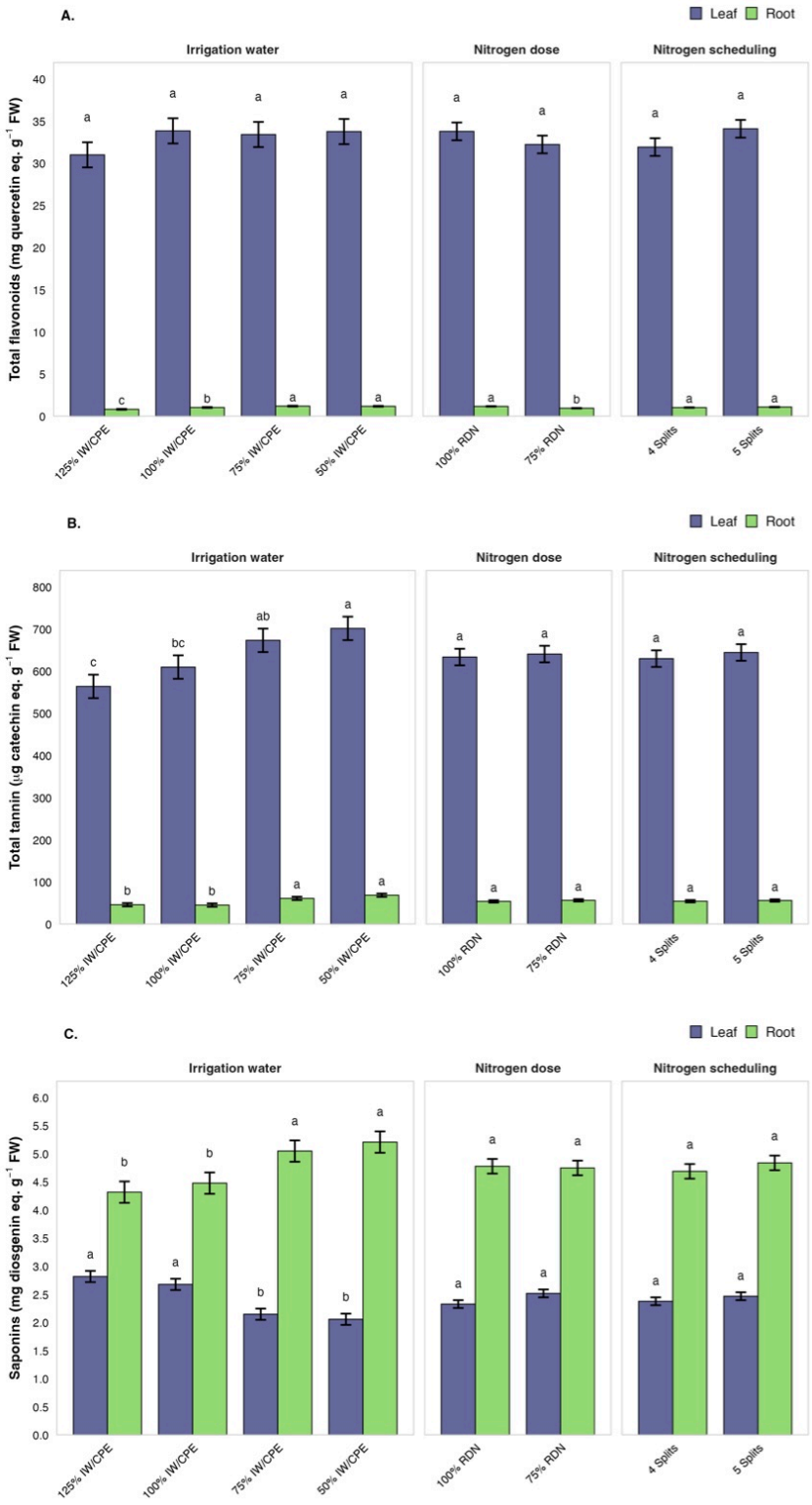

Figure 6

Effect of irrigation levels, nitrogen dose and nitrogen scheduling on total flavonoids (mg quercetin eq. g^{-1} FW), total tannins (µg catechin eq. g^{-1} FW) and saponins (mg diosgenin eq. g^{-1} FW) in fodder beet leaf and root under drip irrigation.

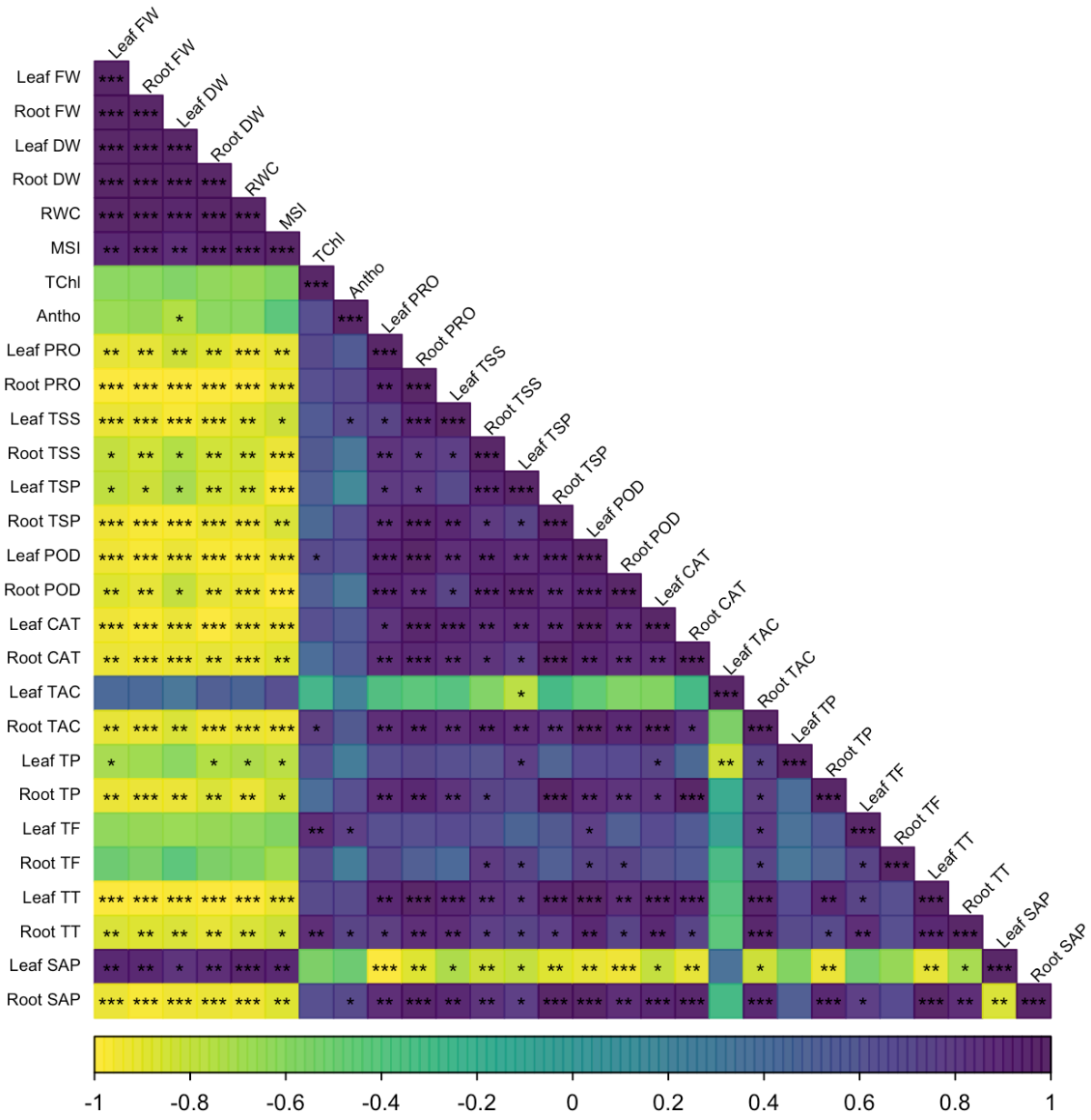

Figure 7

Correlation matrix representing the relationship of morphological and physio-biochemical attributes of fodder beet leaf and root grown with different irrigation water, nitrogen dose and nitrogen scheduling under drip irrigation.

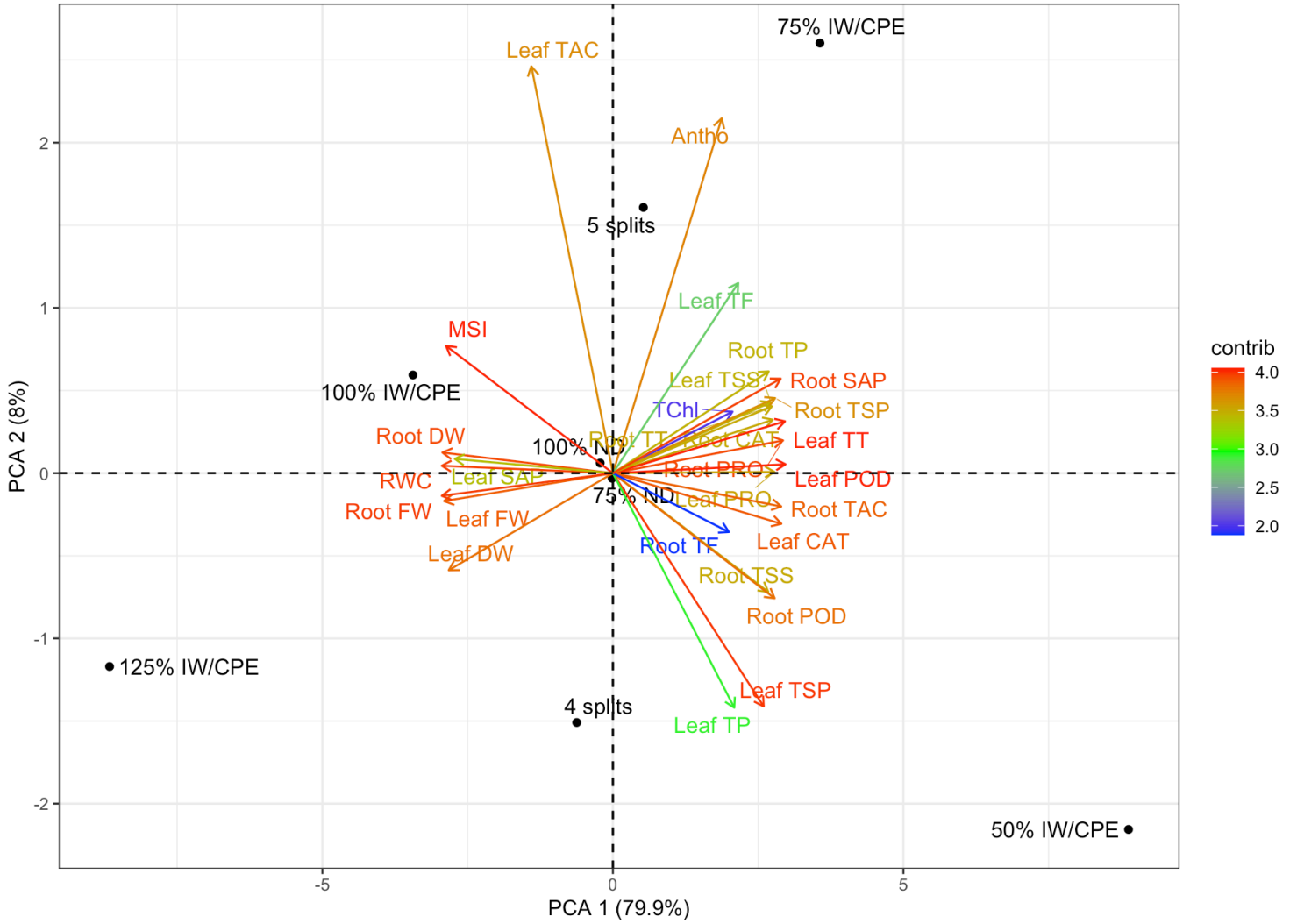

Figure 8

Principal Component Analysis (PCA) biplot of morphological and physio-biochemical attributes in fodder beet showing variable contributions and sample distribution across principal components 1 (PCA 1) and 2 (PCA 2).

Supplementary Files

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- Supplementrydata.docx