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STUDY OF INTERACTIONS BETWEEN FACTORS DETERMINING ABIOTIC
STRESS TOLERANCE, PRODUCTIVITY, AND QUALITY PARAMETERS OF
ALFALFA GENOTYPES

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ABIOTIC STRESS TOLERANCE, PRODUCTIVITY, AND QUALITY
PARAMETERS OF ALFALFA GENOTYPES**

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1. INTRODUCTION

1.1 Background

Alfalfa, also known as the “queen of forage crops” (*Medicago sativa* L.) is among the most valuable perennial leguminous crops cultivated for forage in addition to cereal crops such as maize and rye (Jabessa and Bekele, 2021; Latif et al., 2023). Across the globe, alfalfa is the most cultivated leguminous forage crop due to its high biomass yield, rich protein content, and superior digestibility, which make it an indispensable feed source for livestock. The crop is known for its good health benefits, including cholesterol-lowering effects and use as animal feed (Wang et al., 2024). In Hungary, *Medicago sativa* occupies approximately 216,000 hectares, reflecting nearly 5.2% of the country's arable land area (KSH, 2024). The growing of alfalfa promotes socioeconomic life as the main feed crop supporting beef and dairy industries (Várhidi et al., 2022). Beyond its nutritional qualities, alfalfa also contributes substantially to sustainable agricultural systems through its nitrogen-fixing ability, improvement of soil fertility, and provision of ground cover that reduces erosion and enhances soil structure (Fernandez et al., 2019; Jena et al., 2022). These characteristics make alfalfa one of the most significant crops in both intensive and sustainable livestock production systems.

Despite the agronomic importance, alfalfa cultivation faces increasing challenges from abiotic stresses, among which drought is the most critical. Drought is regarded as a primary limiting factor in agricultural productivity globally, responsible for substantial yield losses in both food and forage crops (Leng and Hall, 2019; Dietz et al., 2021). In alfalfa, drought not only reduces biomass accumulation but also impairs plant persistence, stand longevity, and nutritive value (Liu et al., 2018a; Putnam, 2021; Appiah et al., 2024b). The physiological mechanisms underlying drought-induced damage include reduced photosynthetic efficiency, limited root water uptake, oxidative stress, and impairment of nitrogen fixation processes (Mouradi et al., 2017; Daud et al., 2025). Furthermore, the increasing frequency and severity of drought incidence associated with climate change have become a major concern regarding the long-term sustainability of alfalfa production in water-limited environments.

Crop resilience to drought depends on a combination of genetic and management-related factors. In terms of genetic aspects, cultivars of alfalfa differ widely in their drought tolerance due to variations in traits such as root depth, water-use efficiency, osmotic adjustment, and antioxidative defence mechanisms (Prince et al., 2022; Peng et al., 2025;

Zhang et al., 2025). Consequently, selection of appropriate cultivars, therefore, plays a crucial role in maintaining productivity and quality under water-limited conditions. In addition to genetic improvement, agronomic interventions aimed to alleviate the adverse effects of abiotic stress on crop productivity are currently receiving great attention.

Among these interventions, biostimulants have emerged as promising tools for improving crop resilience under abiotic stress conditions. Biostimulants are a diverse group of substances and microorganisms that stimulate natural processes, thereby enhancing nutrient uptake, stress tolerance, and overall crop performance (Calvo et al., 2014; Du Jardin, 2015; Rouphael and Colla, 2020). Unlike fertilizers, biostimulants do not directly supply nutrients; rather, they act by modulating physiological, biochemical, and molecular processes in plants. Previous studies have shown that biostimulants can improve plant tolerance to drought by enhancing water-use efficiency, stimulating root development, regulating hormonal balance, and increasing antioxidant activity (Anli et al., 2022; Turan et al., 2023; Ahmad et al., 2025). Beneficial microorganisms such as *Trichoderma spp.* have been demonstrated to produce enzymes, antioxidants, and hormones (auxin and gibberellins) that promote growth and resistance to abiotic stress (Csótó et al., 2023). Although the effects of cultivar selection and biostimulant application on drought tolerance have been studied separately, there is limited information available regarding their potential interactive effects, particularly in alfalfa cultivation. It is reasonable to hypothesize that the benefits of biostimulants may vary depending on the genetic background of the crop and, conversely, that the performance of genotypes under stress may be influenced by the presence or absence of biostimulant treatments. Investigating these interactions is crucial for identifying genotype-specific responses, optimizing biostimulant use and developing integrated strategies that optimize both productivity and quality of alfalfa under drought conditions.

1.2. Main Objective

The main aim of this study is to investigate the interaction between biostimulants and alfalfa crop genotypes in determining drought tolerance, productivity, and quality parameters of alfalfa under field conditions.

1.3. Specific Objectives

1. To evaluate the physiological and morphological response of selected alfalfa genotypes to biostimulant application under drought stress.

2. To assess the changes in forage quality traits under drought stress to biostimulants application
3. To investigate the interactive effects of genotype x biostimulant on dry matter yield under drought conditions.
4. To identify genotype x biostimulant combinations with the best performance under drought stress for potential application in sustainable forage production systems.

2. LITERATURE REVIEW

2.1 Overview of alfalfa (*Medicago sativa* L.) and variegated alfalfa (*Medicago x varia* Martyn)

2.1.1 Historical background, botanical characteristics and global significance

Alfalfa (*Medicago sativa* L.) is a perennial herbaceous legume, globally recognized for its exceptional nutritional profile, remarkable yield potential, and robust adaptability across diverse agro-climatic zones (Zhao et al., 2021). Its production history dates back to ancient civilizations where it was lauded for its capacity in supporting agricultural systems and sustaining livestock production (Harouna et al., 2019). The crop is believed to be originated in the Fertile Crescent, specifically in the region encompassing modern-day Iran, alfalfa's cultivation rapidly diffused across Eurasia, propelled by its demonstrated efficacy in improving animal health and productivity (Singh et al., 2007). Alfalfa belongs to the genus *Medicago*, encompassing more than 80 species classified as annual or perennial legumes primarily adapted to temperate climates (El-Ramady et al., 2020). Species in this genus are distinguished by their ability to form symbiotic associations with rhizobial bacteria, which facilitates effective biological nitrogen fixation and substantially enhances soil fertility. Among these species, *Medicago sativa* L. is the most extensively cultivated, valued for its exceptional forage yield, superior nutritional profile, and wide-ranging environmental adaptability. In contrast, *Medicago x varia* Martyn a hybrid derived from crosses between *M. sativa* and *Medicago sativa* subsp. *falcata* displays superior cold hardiness, persistence, and stress tolerance, rendering it well-suited to regions with severe winter conditions (Parajuli, 2023; Tucker et al., 2024).

The botanical attributes of alfalfa are characterized by a deep taproot system, enabling access to water and nutrients from considerable soil depths, thereby conferring resilience to drought conditions (Chamblee, 2013). Its trifoliate leaves, arranged alternately along the stem, contribute to efficient photosynthetic activity, translating into high biomass production. The flowers, characteristically purple and arranged in racemose inflorescences, are predominantly cross-pollinated by insects, particularly bees, emphasizing the vital role of pollinators in seed production and genetic diversity.

One major characteristic of alfalfa is its symbiotic relationship with rhizobia bacteria, which enables the fixation of atmospheric nitrogen into the soil. This process reduces the reliance on synthetic nitrogen fertilizers, contributing significantly to sustainable agricultural systems (Miller et al., 2020). Alfalfa also enhances soil fertility, improves soil structure, and supports environmentally sustainable farming practices. Furthermore,

reducing nitrogen fertilizer application, alfalfa production indirectly mitigates nitrous oxide emissions, a potent greenhouse gas, into the atmosphere (Gaffey et al., 2023).

Globally, alfalfa ranks among the foremost forage crops thanks to its high protein content and digestibility, making it a key component of ruminant nutrition to increase milk production and improve animal health. In addition to direct use as forage, it is frequently incorporated into crop rotation systems, where it improves soil health, disrupts pest and disease cycles, and enhances water infiltration (Dixon et al., 2005; Fernandez et al., 2019; Putnam, 2021). Collectively, attributes underscore the crop's enduring global significance in both agricultural productivity and environmental sustainability.

2.1.2 Global production trends and utilisation of the alfalfa crop

Alfalfa cultivation is globally significant yet characterised by shifting production dynamics driven by environmental, economic and technological factors. Alfalfa occupies approximately 30 million hectares globally, with production concentrated in major areas such as North America, Europe, Asia and Africa (Zhou et al., 2021). Despite a relatively stable acreage globally, shift in cultivation trends are evident due to high demand for high-quality forage, particularly driven by the expansion of the livestock industries. The distribution of alfalfa production is highly uneven, with North America dominating the global output accounted for 41%, followed by Europe with 25%, South America with 23%, Asia with 8% whereas Africa and Oceania accounted for 3% (Yuegao et al., 2009). This prominence is attributed to advanced production systems, favorable climatic conditions, and strong integration with livestock industries. In contrast, emerging production regions are expanding cultivation to meet growing domestic demand for animal feed.

In terms of utilisations, alfalfa is valued as a fodder crop for dairy, beef, and equine industries (Fernandez et al., 2019; Ijaz et al., 2021). It is commonly processed into hay, silage, and pellets and is also a significant commodity in international forage markets. Its high crude protein content and digestibility make it particularly suitable for enhancing livestock productivity and supporting efficient animal nutrition (Radovic et al., 2009; Oukaltouma et al., 2021). Furthermore, alfalfa has minor applications in human consumption, such as sprouts and dietary supplements; its primary economic importance remains closely linked to livestock production.

2.1.3 Alfalfa production in Hungary

Alfalfa is believed to be introduced into the territory of present-day Hungary via the westward diffusion of the crop from Persia through Greece and into the Roman Empire,

which facilitated its spread across Central Europe, including the Carpathian Basin (Brought et al., 1977). Despite its early establishment, widespread cultivation only occurred in the 18th and 19th centuries, driven by the need to improve livestock production and enhance soil fertility in the region (Julier et al., 2017). Today, alfalfa is a cornerstone of Hungarian agriculture, occupying approximately 8.4% of the country's arable land (KSH, 2025). Its cultivation is concentrated in counties with significant ruminant livestock, such as Bács-Kiskun and Hajdú-Bihar, where it serves as a crucial forage crop for dairy cattle and sheep (Várhidi et al., 2022; KSH, 2025). This spatial distribution reflects the close integration between forage production and livestock systems within the national agricultural framework.

However, recent evidence on production trends reveals notable divergence between the expansion of cultivated area and overall productivity (Appiah et al., 2025). In Hungary, the harvested area for alfalfa increased by 192.7% from 2020 to 2024 relative to red clover, silage, and green maize (**Figure 1**).

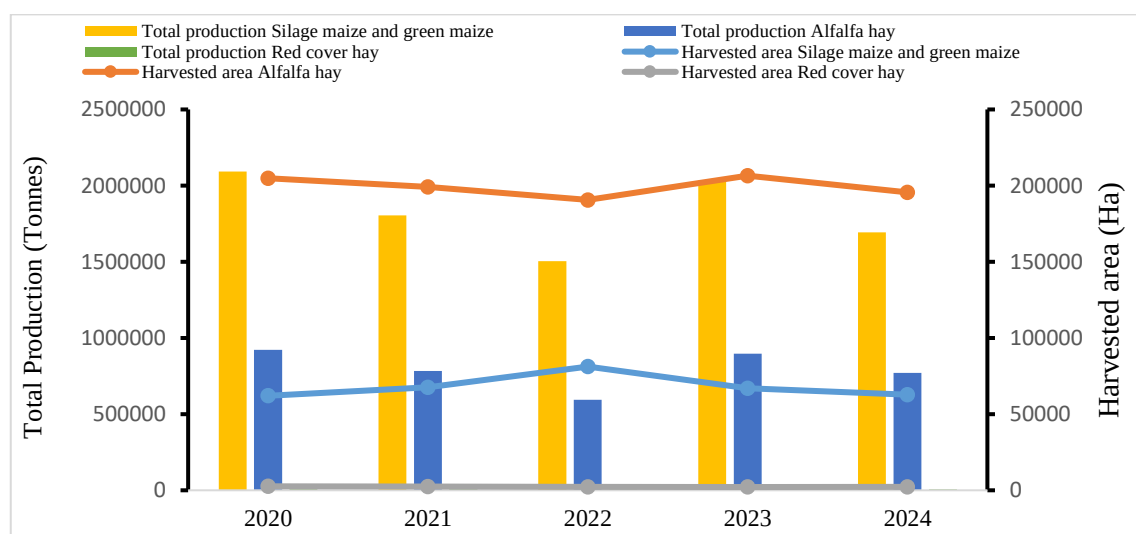


Figure 1: Changes in alfalfa total production and harvested area trends compared with other forage crops in Hungary (KSH, 2024)

In contrast, the total harvested production of alfalfa hay has sharply declined by 56.5% over the same period, accompanied by an 85.4% reduction in average yield per unit area. This dissociation between area expansion and yield performance underscores substantial production inefficiencies in the sector.

This disparity may be explained by multiple factors. Cultivation expansion is likely occurring on marginal lands or lower-quality soils, thereby constraining yield potential.

Additionally, heightened exposure to abiotic stresses, particularly drought, may be limiting productivity. Moreover, deteriorating soil fertility and suboptimal management practices could further intensify yield constraints. Collectively, these factors indicate that the growth in cultivated area has not been matched by equivalent advances in agronomic performance.

2.1.4 The climatic requirement and adaptability of alfalfa and variegated alfalfa

The agro-economic success of alfalfa is predicated on its ability to adapt to sub-optimal environmental conditions, with drought tolerance being a paramount trait for cultivation in water-limited regions. Optimal alfalfa growth is achieved in deep, well-drained loamy soils with a pH of 6.5-7.5 under average daily temperatures of 25°C and annual precipitation ranging from 600–1200 mm (Armah-Agyeman et al., 2002; Bansal and Yin, 2021). However, a key attribute of the species is its ability to persist beyond these optimal conditions, which underpins its widespread cultivation across diverse agro-ecological zones. This adaptability is facilitated by a deep taproot system that allows access to deep soil moisture from deeper soil layers and supports survival under drought conditions. As a result, alfalfa can persist in arid environments receiving as little as 200 mm of annual rainfall under marginal conditions. However, this survival strategy incurs a significant agronomic penalty: substantial reductions in dry matter yield and a frequent decline in forage quality due to increased fibre content (NDF, ADF) (Peterson et al., 1992; Liu et al., 2018). These responses highlight the sensitivity of productivity to environmental stress, even in a relatively resilient species.

Alfalfa exhibits a wide range of tolerance to abiotic stresses, including drought, cold and heat, although the extent of tolerance is highly genotype-dependent. The species is capable of undergoing cold acclimation and becoming dormant to survive freezing winter temperatures, though susceptibility to winter injury varies considerably among cultivars (McKenzie et al., 1998). Conversely, prolonged exposure to high temperatures (>35°C) typically inhibits growth and shortens stand longevity. These contrasting responses underscore the importance of climatic factors in determining both the productivity and longevity of alfalfa stands.

The genetic variation in the *Medicago* genus provides an important basis for adaptation to diverse environments. Especially *Medicago x varia* Martyn (variegated alfalfa), a hybrid derived from crosses between *M. sativa* and *Medicago sativa* subsp. *falcata*, exhibits enhanced tolerance to cold and drought stress compared to conventional *M. sativa* cultivars (Hrbáčková et al., 2020; He et al., 2022). Whereas *M. sativa* is generally

associated with higher yield potential under favourable conditions, *M. x varia* demonstrate greater persistence and stability under environmental stress, making it particularly suitable for regions with harsh winters and variable climatic conditions.

Overall, the adaptability of alfalfa to a broad range of climatic conditions reflects a combination of physiological traits and genetic diversity. However, deviations from optimal environmental conditions often result in reduced productivity, highlighting the need to consider both climatic suitability and genotype selection in the establishment and management of alfalfa-based systems. **Table 1** summarises the optimal climatic conditions for selected forage crops.

Table 1: Annual climatic conditions for optimum growth of some selected forage crops

Forage crops	Annual temperature (°C)	Annual rainfall (mm)
Alfalfa	22.2 - 24.4	508 -1168.4
Red clover	20 - 25	600 - 700
White Clover	4.3 - 21.8	900 - 1300
Birdsfoot trefoil	7.0 - 16.9	240 - 255
Pinto peanut	21-22	1500

2.2 Abiotic stress in plants: an overview

Abiotic stress is defined as the adverse effect of non-living environmental factors such as drought, salinity, and temperature extremes on plant growth, development, and ultimately, productivity. These stresses represent major constraints to global agricultural production and are exacerbated by climate change (Erice et al., 2009; Baweja and Kumar, 2021; Thakur et al., 2021). Amongst these stresses, drought represents the most significant abiotic challenge, often compounded by concurrent heat stress, thereby amplifying its impacts on plant performance. The detrimental effects of these abiotic stresses are primarily at physiological and biochemical levels. A key initial response to water deficit is stomata closure, which inadvertently reduces transpirational water loss but simultaneously restricts carbon dioxide (CO₂) uptake (Hou et al., 2022). This restriction of CO₂ fixation leads to an energy imbalance within the photosynthetic apparatus, particularly in the chloroplasts, resulting in the over-production of reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide (Muhammad et al., 2021; Sachdev et al., 2023). At high concentrations, ROS cause oxidative damage, leading to lipid peroxidation, protein denaturation, and nucleic acid mutation, which

disrupt cellular homeostasis and can induce cell death (Muhammad et al., 2021). To mitigate this damage, plants have evolved a range of adaptive mechanisms to maintain cellular homeostasis. These can be broadly categorized as (1) avoidance mechanisms, such as deep root systems for water access; and (2) tolerance mechanisms, which include the upregulation of antioxidant enzymes (superoxide dismutase, catalase, and peroxidase) to scavenge ROS and the synthesis of compatible osmolytes (proline, glycine betaine) to maintain cellular turgor and protect macromolecules (Devi et al., 2017; Nawaz et al., 2023). Overall, the plant response to abiotic stress involves a complex cascade of events, from stress perception to physiological and biochemical adaptation. Understanding these mechanisms is essential for explaining variations in plant performance under adverse environmental conditions and provides a foundation for further research into stress resilience in crop species.

2.2.1 Drought stress

Drought stress, fundamentally a condition of water deficit, occurs when the rate of plant transpiration exceeds its rate of water uptake from the soil, leading to a critical loss of cellular turgor pressure. While often triggered by a lack of precipitation, the severity of drought is frequently exacerbated by high temperatures and wind, which accelerate soil water evaporation and plant transpirational losses (Farooq et al., 2009). The magnitude of the stress is determined by its intensity, duration, and the developmental stage of the plant during the water shortage. The immediate physiological consequence of drought is a reduction in cell turgor, which directly inhibits cell expansion and root and shoot growth, making growth one of the most drought-sensitive processes (Pugnaire et al., 1999). To further minimise water loss, plants respond through stomatal closure; however, this adaptive response restricts the influx of carbon dioxide (CO₂) into the leaf mesophyll, leading to a sharp decline in photosynthetic carbon assimilation (Agurla et al., 2018; Ashkavand et al., 2018). As described in Section 2.2, such limitations in photosynthesis can disrupt the balance of the photosynthetic apparatus and promote the accumulation of reactive oxygen species (ROS), resulting in oxidative stress and cellular damage. Furthermore, drought disrupts the transport of nutrients and assimilates throughout the plant, as water is the essential medium for their movement, which further constrains growth and metabolic processes (Farooq et al., 2009). The combined impact of these physiological limitations ultimately leads to significant reductions in biomass accumulation and overall crop productivity.

In the case of alfalfa, a crop valued for its high biomass, these physiological disruptions induced by drought have direct implications on both yield and forage quality. Water deficit reduces herbage yield and can significantly alter key forage quality parameters, such as increasing fibre content and reducing digestibility. These changes negatively affect the nutritional value of the crop for livestock, thereby reducing its overall agronomic and economic performance.

2.2.2 Temperature stress

Temperature stress, resulting from exposure to heat or cold temperatures, is a major abiotic constraint that disrupts cellular homeostasis, impairing plant metabolism, growth, and ultimately productivity. The effects of temperature stress are particularly acute for perennial crops like alfalfa, which must endure seasonal temperature fluctuations over multiple years. High-temperature stress is defined as an exposure to temperatures above a critical threshold (typically $>35^{\circ}\text{C}$ for alfalfa) for a duration sufficient to cause irreversible damage to growth and development (Wahid, 2007). Heat stress negatively impacts plant functions, including enzyme activity, membrane stability, and metabolic processes. It can denature enzymes and destabilise membranes, with severe consequences for nitrogen metabolism and protein integrity, a critical issue for a high-protein forage crop. Furthermore, high temperatures accelerate water loss through transpiration, often inducing a secondary drought stress even when soil moisture is adequate (Takahashi et al., 2013; Chandrakar et al., 2018). In addition, heat stress can alter developmental patterns, such as promoting hypocotyl elongation and inducing early flowering, processes mediated by phytohormonal shifts involving auxins and ethylene (Fei et al., 2018). These disruptions collectively lead to reduced biomass accumulation and can compromise forage quality.

Low-temperature stress is categorised as chilling stress ($0\text{--}15^{\circ}\text{C}$) or freezing stress ($<0^{\circ}\text{C}$). Chilling stress damages plants without ice crystal formation, while freezing stress causes extracellular ice crystallisation. This process lowers the extracellular water potential and draws water out of cells, leading to lethal intracellular dehydration (Hasanuzzaman et al., 2013). The primary site of injury is the cellular membrane, where reduced temperature decreases membrane fluidity, increases rigidity, and disrupts permeability. These changes result in electrolyte leakage and a loss of cellular compartmentalisation (Lukatkin, 2003; Yadav, 2010). This membrane damage disrupts the function of membrane-bound proteins and organelles, negatively impacting

respiration and photosynthesis (Niu and Xiang, 2018; Samanta and Roychoudhury, 2022).

In alfalfa, temperature stress has direct implications for both productivity and stand persistence. Heat stress during the summer can suppress biomass accumulation and negatively affect forage quality, particularly protein concentration. Conversely, exposure to low temperatures, especially unseasonal frosts can damage sensitive regrowth, impacting stand longevity and seasonal productivity. These responses highlight the importance of temperature as a key environmental factor influencing the performance and stability of alfalfa production systems.

2.3 Drought stress and its impact on alfalfa growth and productivity

Drought stress is one of the major limiting factors in alfalfa cultivation, resulting in reduced growth and development and biomass accumulation. These effects are manifested via morphological changes such as reduced plant height and diminished leaf area, which are physiological disruptions associated with water deficit. As outlined in Section 2.2.1, drought-induced reductions in cellular turgor and photosynthetic capacity play a central role in constraining plant development.

2.3.1 Plant height and stem elongation

Drought stress consistently inhibits plant height, a key visual indicator of alfalfa's response to water deficit (Luo et al., 2020; Bhattacharya, 2021a; Nour et al., 2024). This reduction in growth is a direct morphological reflection of impaired cellular processes due to loss of turgor pressure, which is the fundamental force for cell expansion, which limits internode elongation and overall stem growth (Liu et al., 2018a; Yang et al., 2023). In addition to this biophysical limitation, drought disrupts critical hormonal signalling pathways via suppression of gibberellins, which are primary promoters of stem elongation (Farooq et al., 2008). Furthermore, another critical factor contributing to reduced growth in alfalfa under drought stress conditions is the inhibition of symbiotic nitrogen fixation (Mishra et al., 2024; Wang et al., 2024). Inadequate nitrogen availability further inhibits the synthesis of amino acids and proteins, thereby exacerbating the reduction in growth processes and overall biomass accumulation (Wahab et al., 2022). These limitations together result in poor growth, lower canopy height and reduced forage yield at harvest. The severity of the impact is influenced by the timing, duration, and intensity of the drought stress, as well as genetic variation among cultivars, highlighting the importance of selecting drought-resilient varieties that can better maintain growth under water-limited conditions.

2.3.2 Leaf area index

Drought stress significantly reduces leaf area index (LAI), which directly impacts photosynthetic capacity in alfalfa (Luo et al., 2020). This reduction in LAI is achieved through two primary mechanisms: inhibition of new leaf expansion and accelerated senescence of existing leaves. Loss of turgor pressure limits leaf expansion, resulting in smaller leaf size, whereas drought-induced hormonal changes promote the breakdown of older leaves and the remobilisation of nutrients (Wahab et al., 2022; Mouradi et al., 2022). This prioritisation of resources enhances the plant's chances of survival at the expense of its total photosynthetic area. The agronomic consequences of a lowered LAI are profound. A reduced canopy surface area directly translates to decreased light interception, limiting the energy available for photosynthesis and ultimately compromising biomass accumulation and yield (Niinemets, 2007). Furthermore, changes in canopy structure can alter microclimatic conditions within the stand, which can influence the growth dynamics of individual plants and the overall productivity of the field (Niinemets, 2010). These effects collectively contribute to reduced yield and compromised forage quality under drought conditions. Therefore, the ability to maintain a functional LAI under moderate stress, or to rapidly regenerate leaf area upon re-watering, is a critical trait for drought-resilient alfalfa varieties.

2.3.3 Normalised difference vegetation index (NDVI)

NDVI, a widely used remote sensing index derived from the normalized difference between near-infrared and red reflectance, effectively reflects vegetation vigor, chlorophyll content, and photosynthetic activity by integrating biomass, leaf area index, and canopy greenness. Under optimal water conditions, high NDVI values in alfalfa are typically associated with dense canopy cover and active photosynthesis (Condorelli et al., 2018; Prince et al., 2022; Koc et al., 2023). In contrast, drought typically causes NDVI to decline due to reductions in canopy vigor, LAI, and photosynthetic efficiency. These changes alter spectral reflectance patterns by decreasing NIR reflectance and reducing red light absorption, resulting in lower NDVI values (Bellague et al., 2016; Koc et al., 2023; Daud et al., 2025). The extent of NDVI reduction is affected by stress intensity, duration, and plant developmental stage, with greater declines under severe or prolonged drought conditions. NDVI effectively predicts the significant effects that drought imposes on plant growth and physiological processes, making it a reliable metric for monitoring vegetation stress in alfalfa production systems, particularly in water-limited environments (Liu et al., 2018 b; Olii et al., 2021).

2.3.4 Biomass accumulation

Biomass accumulation in alfalfa is severely constrained by drought stress, indicating the cumulative effects of multiple physiological and biochemical disruptions. The decline in harvestable yield primarily results from limited carbon assimilation, impaired nutrient relations, and direct cellular damage. Drought-induced stomatal closure limits carbon dioxide uptake, thereby reducing photosynthetic carbon fixation. This restriction is further compounded by reductions in leaf area index, which limit light interception (Smith et al., 2019; Kapoor et al., 2020). Concurrently, the cumulative effect is a drastic reduction in the production of photoassimilates, the fundamental building blocks for biomass. Drought stress also disrupts nutrient uptake and assimilation (Farooq et al., 2009). As the soil water potential drops, the mass flow of dissolved nutrients to the roots is diminished, leading to deficiencies in key elements like nitrogen, phosphorus, and potassium (Bhattacharya, 2021b). In alfalfa, this is critically exacerbated by the inhibition of symbiotic nitrogen fixation under water stress, which results in reduction of nitrogen availability for protein synthesis and growth processes (Mishra et al., 2024; Wang et al., 2024). In addition, drought-induced oxidative stress can damage cellular structures, including chloroplast membranes, lipids, and proteins, impairing the very machinery responsible for growth and defense (Mansour et al., 2021; Rad et al., 2021). The plant must then divert energy and resources from growth towards antioxidant defense and cellular repair, representing a significant metabolic cost that further depletes biomass potential. The combined impact of these factors results in a substantial decline in biomass accumulation, with the extent of reduction depending on stress intensity, duration, and the genotype's ability to mitigate these three cascading limitations.

2.3.5 Photosynthesis and gas exchange

Drought stress induces a severe inhibition of photosynthesis in alfalfa, a primary physiological driver behind the observed reductions in growth and yield. The impairment of this process is characterized by a sequential progression from stomatal limitations to more damaging non-stomatal limitations, ultimately determining the plant's capacity for carbon assimilation. The initial and most rapid response to water deficit is stomatal closure a protective measure to reduce transpiration. This closure also limits the diffusion of carbon dioxide (CO₂) into the leaf, thereby decreasing the rate of photosynthetic carbon assimilation (Dias and Brüggemann, 2010; Staniak et al., 2018). However, prolonged drought unveils more profound non-stomatal limitations. As stress persists, photosynthetic machinery sustains damage. These damages include a decline in

chlorophyll content, disruption of thylakoid membranes, and the decreased activity of key photosynthetic enzymes such as Rubisco (Iqbal et al., 2019; Mouradi et al., 2022). These effects reduce the efficiency of photosynthetic apparatus and limit the plant's capacity to make good use of absorbed light energy.

Pioneering studies by Antolin and Sanchez-Diaz (1993) showed that even after rewatering restored stomatal conductance, photosynthetic rates remained markedly suppressed, indicating lasting metabolic and ultrastructural damage to chloroplasts. More recent research attributes this persistent suppression to oxidative damage in photosystem II and impaired RuBP regeneration. A key physiological trade-off under drought concerns water-use efficiency. Although overall photosynthesis declines, intrinsic leaf-level WUE often improves, allowing greater biomass production per unit of water (Abid et al., 2016). This response is dynamic; for example, partial root-zone drying can maintain net photosynthesis while reducing stomatal conductance and transpiration, thereby enhancing WUE without always compromising yield in some crops (Zhang et al., 2019a).

2.3.6 Drought stress effects on forage yield and quality

2.3.6.1 Forage yield

Drought conditions consistently lead to a reduction in forage dry matter yield across various species, with the severity and timing of water deficit significantly influencing the extent of this reduction (Liu et al., 2018a). The decline in dry matter yield of alfalfa under water deficit is associated with reduced plant height and biomass accumulation due to limitations in photosynthesis and resource availability (Fahad et al., 2017; Luo et al., 2020). Yield losses of 55% have been reported for alfalfa cultivars subjected to water deficit conditions, underscoring the substantial economic impact of drought on sustainable alfalfa production (Peterson et al., 1992; Leng and Hall, 2019; Baral et al., 2022; Liu et al., 2023). Beyond direct yield reduction, drought can also induce changes in the plant's physiological responses, such as delayed leaf senescence and increased root growth, which may indirectly affect overall biomass production and resource allocation (Kulkarni et al., 2018). Furthermore, drought stress can inhibit tillering and branching, while simultaneously accelerating tiller death and leaf senescence, thereby impacting the structural components that contribute to total forage yield (El Sabagh et al., 2025). The underlying mechanisms for this yield reduction often involve a decrease in the absorption of photosynthetic radiation by the canopy, diminished radiation efficiency, and a reduced harvest index. It postulated that approximately 5 to 7.4 inches of water is required for

optimal alfalfa growth, with deficits below this threshold directly correlating with reduced dry matter production. A recent report by Liu et al. (2023) demonstrated an average dry matter yield loss of 33.2% with losses exceeding 50% under severe drought conditions. Furthermore, Liu et al. (2018a) demonstrated that severe drought stress conditions could reduce yield up to 83%, particularly when occurring in late spring. These findings collectively indicate that alfalfa is a highly susceptible crop to drought, with yield reductions directly proportional to the severity and duration of water deficit.

2.3.6.2 Crude protein content

Drought generally limits overall dry matter production; however, its effect on crude protein content (CP%) in forage species, particularly alfalfa, is more complex and often exhibits an inverse relationship (Shaaban et al., 2023). Specifically, under drought stress, although biomass accumulation is reduced, crude protein concentration can increase due to a reduction in carbohydrate synthesis and a concurrent increase in nitrogenous compounds, which maintains cellular osmotic pressure (Kačienė et al., 2022). This phenomenon is attributed to a greater proportion of leaves, which are richer in protein, relative to stems, during short-term or mild drought events (Lin et al., 2020). However, prolonged or severe drought conditions can ultimately lead to a decrease in crude protein content as nitrogen uptake and assimilation are significantly impaired, and cellular functions are severely disrupted (Wang et al., 2024). Crude protein fractions in alfalfa have been observed to decrease under drought stress (Liu et al., 2017). Similarly, a comprehensive study by Liu et al. (2023) reported an average decrease of 19.7% crude protein under severe drought. In contrast, other studies indicate inconsistent effects on crude protein percentage, where studies report attributing increased crude protein content to concentration effects when carbohydrate accumulation is highly inhibited by nitrogen uptake (Pecetti et al., 2017; Baral et al., 2023). These discrepancies likely reflect differences in drought severity, soil nitrogen availability and harvesting timing. Importantly, even when CP% rises, the total protein yield per hectare invariably decreases due to the much larger reduction in biomass.

2.3.6.3 Fibre concentration

Drought stress typically leads to an increase in fibre components, such as neutral detergent fibre and acid detergent fibre, primarily due to reduced cell wall digestibility and a higher proportion of structural carbohydrates relative to soluble contents as the plant matures prematurely under stress conditions (Liu et al., 2018a; He et al., 2022). This increase in fibre content, particularly lignin, directly correlates with a reduction in forage

digestibility and metabolizable energy for livestock (McDonald et al., 2021). Moderate drought may slow lignification, potentially reducing fibre accumulation (Deetz et al., 1996; Kuchenmeister et al., 2013; Staniak, 2019). However, under severe drought, accelerated lignification and senescence result in a significant increase in fibre content in drought-stressed plants, thereby compromising forage quality (Liu et al., 2018a; Rad et al., 2021; Liu et al., 2023). This is because NDF and ADF are negatively correlated with digestibility in ruminants; thus, increases represent a major reduction in feed value.

2.3.6.4 Soluble sugars and carbohydrates

Water deficit conditions frequently lead to an increase in water-soluble carbohydrates and other non-structural carbohydrates in forage legumes, particularly under moderate stress, as plants accumulate osmolytes for osmotic adjustment (Ressaissi et al., 2023). However, extreme or prolonged drought can deplete these reserves due to inhibited photosynthesis and increased respiration, ultimately reducing the overall concentration of soluble sugars (Zi et al., 2022). Furthermore, the timing of drought can significantly influence the carbohydrate partitioning and accumulation, with early-season drought often having a more pronounced impact on subsequent carbohydrate availability than later-season stress (Wrobel et al., 2022). Severe water deficits have been shown to reduce carbohydrate content in legumes, indicating a shift towards protein deposition when carbohydrate translocation is compromised (Farooq et al., 2018). However, the specific impact on hemicellulose can vary, with some studies indicating stability or even an increase under drought conditions, highlighting the nuanced interaction between drought severity, duration, and plant responses in carbohydrate partitioning.

2.3.6.5 Forage nutrient composition

Drought stress significantly alters the elemental composition of forage crops, impacting the availability and concentration of essential macro and micronutrients critical for animal health and productivity. This alteration is primarily mediated by changes in nutrient uptake efficiency, translocation within the plant, and concentration effects due to reduced biomass accumulation (Izidro et al., 2024). Uptake and assimilation of nitrogen (N) are often reduced under water deficit conditions, leading to lower crude protein content in severe cases, as discussed previously (Esmaily et al., 2024). Conversely, some studies suggest that certain elements like phosphorus may become more concentrated in tissues due to reduced plant growth, although their overall acquisition from the soil might still be impaired (Dumont et al., 2014). Beyond nitrogen and phosphorus, the uptake and distribution of potassium, calcium, magnesium, and various micronutrients like zinc and

copper are also critically affected by drought, with studies indicating both increased concentration due to biomass reduction and decreased total accumulation due to impaired root activity and soil moisture-nutrient interactions (AbdElgawad et al., 2014). In addition, potassium (K) may increase or remain stable due to its role in osmotic adjustment (Abid et al., 2016). The accumulation of osmolytes (e.g., proline) supports plant survival but does not contribute meaningfully to livestock nutrition. Overall, this evidence clearly shows a challenge; drought simultaneously reduces alfalfa yield and quality traits by increasing fibrous content and often decreasing protein concentrations. The ultimate impact on quality, whether digestibility improves or declines, is contingent on the intensity and timing of the drought stress. This yield-quality dynamic underscores the necessity of sustainable nutrient management (biostimulant) and genetic strategies that aim to mitigate the yield losses of drought, rather than relying on a potential concentration effect that offers little economic consolation to producers.

2.4 Genetic factors influencing drought tolerance in alfalfa

The development of drought-tolerant alfalfa varieties is a critical breeding objective, yet it remains a critical challenge due to the polygenic nature of the trait and its significant interaction with environmental variables (G x E) (Annicchiarico et al., 2016; Valliyodan et al., 2016). Despite this complexity, substantial genetic variation for drought resilience exists within the alfalfa germplasm, particularly in accessions and wild relatives (*M. sativa subsp. falcata*) originating from arid environments, providing a foundational resource for genetic improvement (He et al., 2022). This diversity provides an important resource for improving drought resilience through breeding.

Genetic variation in drought tolerance is commonly associated with key physiological and morphological traits that influence plant performance under water-limited conditions. Parameters/traits such as water-use efficiency (WUE), stomatal conductance, osmotic adjustment, and root architecture have been well-documented in forage grasses and legumes, including alfalfa, and these traits are heritable and can be selected for in breeding programmes (Abberton et al., 2008; Khazaei et al., 2014; Abid et al., 2016; Sallam et al., 2019; Lopez et al., 2019). These parameters or traits have shown to improve the plant's ability to maintain growth and productivity under drought stress and are often used as indirect selection criteria.

Modern genomics has provided powerful tools to move beyond phenotypic selection particularly enhancing the ability to identify and utilise genetic determinants of drought

tolerance. Molecular marker-assisted selection (MAS) has been employed to identify quantitative trait loci (QTLs) associated with drought tolerance components in alfalfa (Varshney et al., 2021). For instance, QTLs linked to deeper root systems, a key drought-avoidance trait, have been mapped, offering a pathway for marker-assisted breeding to enhance soil water exploration. Furthermore, gene expression studies (transcriptomics) have begun to unravel the intricate molecular networks underpinning alfalfa's drought response particularly those involved in osmotic regulation, antioxidant defence, and stress signalling pathways (Daud et al., 2025; Peng et al., 2025). These molecular tools complement traditional phenotypic selection by enabling more precise identification of traits linked to drought resilience. However, a significant limitation in breeding for drought tolerance persists in the context-dependency of tolerance mechanisms. A genotype's superior performance is often contingent on the specific type and timing of drought stress (terminal vs. intermittent), making it difficult to breed universally resilient varieties (Iseki et al., 2018; Gonçalves et al., 2019; Putnam et al., 2021). This underscores the necessity for precision in phenotyping and for breeding programmes to select under conditions that reflect the target production environment. Overall, the genetic architecture of drought tolerance in alfalfa is undoubtedly complex; the integration of conventional breeding focused on physiological traits with advanced genomic tools (QTL mapping, transcriptomics) provides a multifaceted strategy to accelerate the development of resilient cultivars. The future of alfalfa breeding lies in leveraging these genetic insights to pyramid favourable alleles for component traits, thereby building genotypes with robust and stable performance across variable water-limited environments.

2.5 Variation in alfalfa varieties: effects on herbage yield, nutritional value, and quality traits

The extensive genetic diversity within the alfalfa (*Medicago sativa* L.) germplasm is a critical resource for breeding and management, particularly in the context of abiotic stress resilience. Varietal differences profoundly influence herbage yield, nutritional value, and most importantly, the stability of these traits under drought conditions (Edwards et al., 2016; Miller et al., 2020). These varietal differences or variability provides the genetic baseline for selection and contributes to differential performance of plants across diverse environments. Recent evidence highlights that the impact of varietal selection on herbage yield under drought is mediated by several key traits (Griffith 1993; El-Ramady et al., 2020). For instance, El-Ramady et al. (2020) reported drought-tolerant varieties often

possess a deeper and more prolific root system, enhancing water foraging from subsoil layers and directly contributing to yield stability under water deficits. Griffith (1993) demonstrated that later-maturing varieties often achieve higher yields in optimal conditions due to a prolonged vegetative period; their longer cycle may increase their exposure to terminal drought. Conversely, early-maturing varieties may escape the worst of a late-season drought but have a lower yield potential. In addition, genetic variation in WUE contributes to differences in productivity under drought, as varieties with the ability to fix more carbon per unit of water transpired will inherently maintain higher yields under limited water availability (Ottman and Putnam, 2017). In contrast, evidence on varietal yield differences is not always consistent. Some comparative study by Liu et al. (2018a) provided direct evidence by comparing 'Gold Queen' and 'Suntory' alfalfa, showing that 'Gold Queen' consistently maintained superior performance under drought. While Shewmaker et al. (2013) note no significant yield difference among cultivars for a given water amount, highlighting that genetic potential can be context-dependent and is not always the primary limiting factor. Overall, these findings support the presence of exploitable genetic variability in yield-related parameters.

The effect of drought on the quality of forage crops is equally subject to significant genotypic variation, which can alter the typical yield-quality trade-off. Under drought stress conditions, differences in morphological traits such as leaf-to-stem ratio preserve a greater proportion of highly digestible, protein-rich tissue, mitigating the drought-induced shift towards lignified, fibrous stems (Marten et al., 1988; Zhang et al., 2023). In addition, varieties with maximum leaves tend to have a higher nutritive value and are more digestible than stems (Heuschele et al., 2023). Varieties differ in their ability to maintain nutrient uptake and translocation (especially nitrogen) under stress, which directly influences crude protein content independent of a concentration effect (Sleugh et al., 2000; Chamblee, 2013). Changes in fibre composition are also genotype-dependent, influencing digestibility and overall feed value. The selection of varieties with inherent drought-avoidance (deep roots) and drought-tolerance (high WUE, osmotic adjustment) mechanisms is the first and most critical step in ensuring productive and sustainable alfalfa cultivation in water-limited environments. Therefore, the choice of alfalfa genotype is not a background variable but a primary determinant of drought resilience. The documented variation in physiological and morphological traits means that the potential response to an external intervention, such as a biostimulant application, will be inherently shaped by the genetic background of the plant. A biostimulant may elicit a

dramatically different response in a drought-sensitive variety compared to a drought-tolerant one, a critical interaction that lies at the heart of this thesis's investigation.

2.6 Biostimulants and alfalfa production under drought stress condition

In the face of increasing drought stress, biostimulants have emerged as a promising agronomic tool for enhancing crop resilience and productivity. The use of novel approaches in the cultivation of crops such as alfalfa (*Medicago sativa* L.) has gained great interest because of the role they play in improving plant resilience, maintaining productivity and supporting resource-use efficiency under water-limited environments. Biostimulants unlike fertilizers acting as a direct nutrient source, function by stimulating natural physiological processes that enhance plant performance. Biostimulants offer a targeted strategy to amplify the plant's innate defence mechanisms (Calvo et al., 2014; Du Jardin, 2015; Samuels et al., 2022; Malécange et al., 2023). The efficacy of these biostimulants in mitigating drought stress in forage grass and legumes such as alfalfa is mediated through a series of interconnected physiological and biochemical pathways (Rouphael and Colla, 2020; Rouphael and Colla, 2021; Ahmad et al., 2025). These interconnected series include the promotion of root growth and soil exploration, enhancement of osmotic adjustment, and stimulation of antioxidant defence systems. Collectively, these responses contribute to improved plant water status, maintenance of metabolic activity, and protection against drought-induced cellular damage.

2.6.1. Definition and classification of biostimulants

Biostimulants represent a category of agricultural inputs designed to enhance plant growth, development, and resilience without acting directly as fertilisers or pesticides (Spinelli et al., 2022). These substances or microorganisms, when applied to plants or the rhizosphere, stimulate natural processes to improve nutrient uptake, increase tolerance to abiotic stresses, and enhance overall crop quality and yield (Calvo et al., 2014; Du Jardin et al., 2015; Samuels et al., 2022). Similarly, the European Union also defines plant biostimulants as products that stimulate plant nutrition processes independently of the product's nutrient content, with the sole aim of improving nutrient use efficiency, tolerance to abiotic stress, quality traits, or availability of confined nutrients in the soil or rhizosphere (García-García et al., 2020). These definitions underscore that biostimulants are not fertilisers, though they may enhance nutrient uptake and utilisation.

Biostimulants are classified into microbial and non-microbial types based on their composition, origin, and mode of action (Calvo et al., 2014; Othibeng et al., 2021). Microbial biostimulants include beneficial bacteria and fungi (plant growth-promoting

rhizobacteria, mycorrhizae fungi, phosphate-solubilising bacteria, and *Trichoderma* spp.) that promote plant growth through various mechanisms, such as nitrogen fixation, phosphate solubilization, and the production of plant growth hormones (Calvo et al., 2014; Drobek et al., 2019; Samuels et al., 2022). Non-microbial biostimulants consist of humic substances, seaweed extracts, protein hydrolysates, and other organic compounds that stimulate plant growth and stress tolerance through diverse physiological and molecular processes (Mrid et al., 2021; Samuels et al., 2022).

However, the rising popularity of these biostimulants in agriculture reflects a growing interest in sustainable and environmentally friendly approaches to crop production (Brown and Saa, 2015). Beyond the effects of plant growth regulators alone, biostimulants are biological formulations that improve plant health and productivity through unique qualities derived from their complex mixes (Rouphael and Colla, 2020; Rouphael and Colla, 2021; Nepthali et al., 2021). Determining the biological basis of biostimulant activity is essential for developing a science-based industry and creating efficient laws. However, because biostimulants come from a variety of sources, such as bacteria, fungi, seaweeds, and humic substances, characterising them is difficult (Yakhin et al., 2017).

2.6.2. Mechanisms of biostimulants under drought conditions

Biostimulants mitigate drought stress via enhancing the key physiological and biochemical defense pathways that improve plant survival and productivity under water-limited environments. Their mechanisms of action can be categorized into a multi-level defence strategy that enhances both drought avoidance and tolerance, thereby maintaining plant function under stress conditions. The initial line of defence involves improving the plant's ability to acquire water, aiding in maintaining cellular homeostasis and the preservation of core physiological functions. Plant based biostimulants such as humic substances and microbial biostimulants including plant growth-promoting rhizobacteria (PGPR), *Trichoderma* spp. enhance root architecture by stimulating the production of root hairs and lateral roots (Canellas et al., 2015; Kamal, 2018; Rouphael and Colla, 2020; Rouphael and Colla, 2021; Tyśkiewicz et al., 2022; Bala et al., 2024). This expansion of the root system increases the surface area for water absorption and allows exploration of a greater soil volume, contributing to greater resilience under drought conditions (Mrid et al., 2021; Tiwari et al., 2023; Zhang et al., 2023). In addition, these biostimulants can improve soil aggregation and water retention capacity in the rhizosphere, creating a more favourable micro-environment for root function.

Biostimulants also contribute to improved cellular water status through the enhancement of osmotic adjustment processes. Plant based biostimulants such as seaweed extracts and protein hydrolysates promote the accumulation of compatible osmolytes like proline and glycine betaine (Manaf, 2016; Rosa et al., 2020; Malécange et al., 2023). This process lowers cellular osmotic potential, helping to maintain turgor pressure essential for cell expansion and metabolic processes under water deficit (Oosten et al., 2017). Furthermore, biostimulants improve antioxidant defense systems by increasing the enzymes' activity, such as reactive oxygen species (ROS) detoxification, thereby reducing the oxidative damage and preserving cellular integrity under stress environments. (Murtic et al., 2019; Zandi and Schnug, 2021; Baltazar et al., 2021; Hasanuzzaman et al., 2021; Rady et al., 2023).

Biostimulants act as hormonal regulations a significant mechanism through which they influence plant response to drought stress. Their functioning in regulating hormones contributes to improved stomatal regulation and delayed senescence by modulating phytohormone balance such as pathways associated with abscisic acid cytokinin (Rampino et al., 2006; Bhupenchandra et al., 2022). Due to these activities, plants are able to balance growth and stress response under adverse stress environments. The combined influence of these mechanisms contributes to preserving essential physiological activities, which lead to maintaining photosynthetic efficiency (De Vasconcelos, 2020; Hasanuzzaman et al., 2021; Baltazar et al., 2021). This is achieved by mitigating stomatal limitations and protecting the photosystem II (PSII) complex from photoinhibition. Consequently, carbon assimilation is sustained, leading to reduced yield losses and better preservation of forage quality under drought conditions (Mackiewicz-Walec and Olszewska, 2016; Zaman et al., 2023; Akdağ et al., 2024). In alfalfa, the efficacy of these mechanisms, however, is influenced by the genetic background of the plant. Different alfalfa varieties may exhibit variable responses to biostimulant application, reflecting differences in their inherent physiological capacity for stress tolerance. This highlights the importance of genotype-dependent responses in determining the overall efficacy of biostimulants under drought conditions.

2.6.3 *Trichoderma* spp. and silicon as biostimulants in sustainable crop production

Trichoderma spp. which is classified as a microbial agent, and non-microbial elements, such as silicon, are among the diverse categories of biostimulants. These biostimulant categories have gained considerable interest due to their vital role in improving crop resilience and performance under abiotic stress conditions, particularly drought.

Trichoderma, a genus of fungi widely distributed in soils, establishes symbiotic relationships with plant roots, colonising the rhizosphere and triggering a range of beneficial effects (Karpushina et al., 2021). These beneficial effects include enhancement of root system architecture, such as increased root length, branching, and root hair development, thereby promoting plant growth (Kamal, 2018; Tyśkiewicz et al., 2022; Fadiji et al., 2022; Csótó et al., 2023). This improvement in root system allows more exploration of the soil profile efficiently and enhances water and nutrient uptake in drought-stressed environments. In addition, *Trichoderma* spp. act as a biological fertilizer which enhances nutrient availability by facilitating the solubilisation of other macronutrients such as phosphorus, thereby increasing plants' nutrient use efficiency (Wijayanto et al., 2022; Bala et al., 2024). Furthermore, the colonisation of plant roots by *Trichoderma* spp. induces substantial physiological changes within the plant, including the activation of stress-responsive pathways that contribute to improved tolerance to drought conditions (Kottb et al., 2015; Fiorentino et al., 2018; Kovács et al., 2021; Csótó et al., 2023). These combined effects can support biomass production and stand persistence of forage crops such as alfalfa under environmental stress.

Silicon, although not considered an essential element for plant growth, is increasingly recognised for its beneficial effects on plant health and abiotic stress tolerance. Silicon is absorbed by plants in the form of silicic acid and deposited in plant tissues, particularly in the cell walls. This forms a protective layer that strengthens structural integrity and reduces transpirational water loss, thereby contributing to improved water-use efficiency under drought conditions (Chanchal-Malhotra et al., 2016; Mandlik et al., 2020; Suparman and Nugroho, 2021). Silicon also plays a role in maintaining physiological stability by supporting photosynthetic performance and reducing the detrimental effects of oxidative stress (Artyszak, 2018; Roychoudhury, 2020; Bhardwaj et al., 2023). Furthermore, silicon-mediated improvements in plant water relations and membrane stability help sustain growth and productivity during periods of water deficit (Rizwan et al., 2015; Thorne et al., 2020; Kovács et al., 2022). In alfalfa and other forage crops, these effects are associated with improved biomass accumulation and better maintenance of forage quality under stress conditions. However, the efficacy of both *Trichoderma* spp. and silicon is affected by environmental conditions and the genetic background of the plant, highlighting the importance of considering their application within specific cropping systems. Their integration into alfalfa production systems represents a

promising strategy for improving drought resilience and supporting sustainable forage production under increasingly variable climatic conditions.

2.6.4 Effect of biostimulant on alfalfa's growth traits under drought stress

Trichoderma spp. has demonstrated a significant capacity to mitigate the negative impact of drought stress on key growth traits in plants. In underwater limited environments, water deficit primarily reduces plant development, which inhibits plant height, leaf area, stem elongation and overall biomass (Brown and Tanner, 1983; Hall, 1993; Prince et al., 2022). However, several evidence demonstrate that the use of *Trichoderma*-based biostimulants has the potential to alleviate these negative effects while promoting overall plant growth under drought stress (Calvo et al., 2014; Du Jardin, 2015; Rouphael and Colla, 2020; Rouphael and Colla, 2021).

In terms of above-ground growth, numerous studies demonstrate that application of biostimulant consistently leads to improvements in growth-related traits such as leaf number, plant height and shoot biomass. Microbial biostimulants particularly *Trichoderma spp.* have been shown to significantly enhance alfalfa growth metrics, revealing a 68.1% increase in shoot dry weight and improved plant vigour compared to the control (Zhang et al., 2019b; Li et al., 2025). Similarly, specific strains of *Sinorhizobium meliloti*, alfalfa's native symbiotic partner, has been associated with increasing plant height, tiller number, and shoot biomass, even under suboptimal conditions (Silva et al., 2020). Under moderate to severe drought, inoculation with *T. harzianum* and *T. asperellum* observed to maintain shoot elongation in maize and wheat, with height increases ranging from 15 to 35% compared to uninoculated stressed controls (Shaheen, 2023; Ascaso et al., 2025). These improvements are associated with enhanced nutrient uptake and efficient physiological functioning under water-limited environments. A robust root system is essential for water foraging under drought. Application of biostimulants have demonstrate to enhance root architecture by increasing root length, density and overall biomass (Sezen and Küçük, 2021; Sezen and Küçük, 2023). A well development roots or expanded roots enabling the plant to access water from a larger soil volume, which is a fundamental drought avoidance strategy. Other studies on cyanobacterial and fungal biostimulants have demonstrated their ability to stimulate root proliferation thereby contributing to drought avoidance (Sezen and Küçük, 2021; Sezen and Küçük, 2023; Xue et al., 2024). The enhancement in both shoot and root together contributes to improve biomass accumulation and yield stability in alfalfa productivity under water deficit environments. Research have shown a well maintained growth

performance and productivity of alfalfa treated with biostimulants compared to untreated groups, reflecting enhanced resilience of the plant under drought stress (Marinova et al., 2023). In addition, microbial biostimulants improve the soil microbiome, enhance soil nutrient availability and enzyme activity, which indirectly supports plant growth, greater biomass accumulation and a higher nutritional index (Zhang et al., 2019b; Wang et al., 2024).

This evidence collectively confirms that biostimulants can effectively promote alfalfa growth under stress. However, a critical limitation in the existing literature is that many studies report the improvements in growth traits without rigorously quantifying the underlying physiological mechanisms in alfalfa under defined drought conditions. Furthermore, the response to biostimulant application is not uniform and may vary depending on the genetic background of the plant. This highlights the importance of considering genotype-dependent responses, particularly the potential interaction between alfalfa genotype x biostimulant treatments. Understanding this interaction is essential for optimising the use of biostimulants in improving drought resilience and forms a key focus of the present study.

2.6.5 Effect of biostimulants on alfalfa's physiological traits under drought stress

Trichoderma-based biostimulants enhance plant's resilience to drought by directly targeting and improving key physiological processes that are critical for survival and productivity under water deficit. Their application leads to measurable improvements in gas exchange, water use efficiency, and cellular protection, effectively mitigating the physiological limitations induced by drought stress (Bulgari et al., 2019; Rouphael and Colla, 2020; Rouphael and Colla, 2021). Drought stress severely inhibits photosynthesis primarily through stomatal closure and damage to the photosynthetic apparatus (Farooq et al., 2009; Zahra et al., 2023). Biostimulants can alleviate these constraints by modulating stomatal behaviour protecting photosynthetic machinery and improving water use efficiency. The application *Trichoderma* spp. have been shown to regulate stomatal aperture, allowing for sustained carbon assimilation under stress condition (Contreras-Cornejo et al., 2014). Conversely, some biostimulants like chitosan can promote stomatal closure under severe stress to conserve water, a form of controlled drought avoidance (Iriti et al., 2009). Biostimulants preserve chlorophyll content and the integrity of chloroplasts. Research emphasises that foliar applications of seaweed extracts and other biostimulants have been demonstrated to inhibit chlorophyll degradation and protect the ultrastructure of chloroplasts, contributing to maintained plant's capacity for

light capture and electron transport under drought stress (Xu et al., 2020; Baltazar et al., 2021). Furthermore, *Trichoderma*-treated plants demonstrate superior stomatal regulation and higher instantaneous water-use efficiency (WUE) under water deficit, resulting in prolonged leaf greenness and delayed wilting (Lllescas et al., 2022). This improvement in WUE optimises balance amongst photosynthetic carbon assimilation and transpiration, thereby maintaining productivity (El Moukhtari et al., 2024). Furthermore, leaf relative water content (RWC) increases ranging from 10 to 25% have been reported across cereal and leguminous crops inoculated with *T. harzianum* under imposed drought (Shukla et al., 2015; Mona et al., 2017). Biostimulants boost the activity of antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase, enhancing the plant's capacity to scavenge reactive oxygen species (ROS) and prevent oxidative damage to membranes and proteins (Choudhary et al., 2017). *Trichoderma*-based biostimulants also stimulate osmotic adjustment by promoting the accumulation of compatible solutes like proline, which helps maintain cell turgor and stabilise proteins and membranes under dehydration stress (Contreras-Cornejo et al., 2015; Wang et al., 2022).

In alfalfa, a legume, the synergy between biostimulants and nitrogen fixation may also influence physiological performance. Co-inoculation with beneficial microbes like specific cyanobacteria (*Nostoc* spp.) and *Sinorhizobium meliloti* can enhance nitrogen availability, which in turn supports the synthesis of chlorophyll and photosynthetic proteins, leading to improved physiological performance under stress (Karaushui et al., 2015). Despite these documented studies convincingly demonstrating the potential of biostimulants to enhance plant's physiological resilience, most of this evidence is derived from controlled environments, so translation of these findings to field conditions remains limited. The transition from pot trials to field conditions introduces complex variables such as soil heterogeneity, microbial competition, and fluctuating environmental stresses. Furthermore, the physiological response is likely modulated by the plant genotype, suggesting that a "one-size-fits-all" biostimulant approach may be ineffective. Therefore, future research must prioritize field-based evaluations that explicitly test the genotype x biostimulant interaction to translate these promising physiological mechanisms into reliable agronomic recommendations for drought-prone alfalfa systems.

2.6.6 Biostimulant effects on forage yield and quality traits under drought stress

The ultimate measure of a *Trichoderma*-based biostimulant's efficacy in forage crop production is its ability to mitigate drought-induced losses in harvestable forage yield and preserve its nutritional quality. Evidence suggests that biostimulants can achieve this by

sustaining the physiological processes that underpin biomass accumulation and nutrient partitioning (Bulgari et al., 2015; Sible et al., 2021; De Beer et al., 2023; El Sabagh et al., 2025).

In terms of forage yield, several studies demonstrate that the application of *Trichoderma*-based biostimulant consistent potential to improve forage crops, such as alfalfa, under stress conditions. Field studies provide the most relevant evidence. For instance, inoculated *Trichoderma* spp. with forage crops, including alfalfa, produced higher dry matter yield, an increase of over 50% over the control under severe drought (Li et al., 2022). Similarly, a three-year field study by Marinova et al. (2023) reported that a vermicompost-based biostimulant significantly increased alfalfa dry matter yield by nearly 5%, indicating improved resilience and productivity over multiple seasons. This yield enhancement is likely the integrated result of the physiological benefits previously outlined: improved water and nutrient uptake from stimulated root systems, sustained photosynthesis, and reduced oxidative damage, which collectively support greater biomass production. Inoculation with beneficial bacteria (*Bacillus* spp.) and fungi (*Arbuscular mycorrhizae*), especially in challenging soils, has been shown to enhance yield by improving soil nutrient availability and plant nutrient status (Cao et al., 2023; Li et al., 2024). However, it is critical to note that biostimulant effects are not universally positive. Research by Písarčík et al. (2019) found that certain biological agents could have inconsistent or even phytotoxic effects, reducing yield and crude protein content in alfalfa. This underscores the importance of product-specific evaluation and highlights that efficacy is highly dependent on the specific biostimulant-plant-environment interaction. In addition to yield, drought often reduces forage quality, which determines the feed's nutritional value of forage and digestibility. However, application of trichoderma-based biostimulant have reported to elucidate these effects. Crude protein content tends to increase with *Trichoderma* application under stress conditions. This improvement is largely attributed to enhanced nitrogen assimilation and microbial activity in the rhizosphere (Singh et al., 2018; Zhang et al., 2019b). Studies on legumes and grasses have consistently reported higher protein levels in treated plants, even under moisture stress conditions (Singh et al., 2018. Akdag et al.,2024).

Fibre content, including neutral detergent fibre (NDF) and acid detergent fibre (ADF), shows variable responses. Some researchers report a slight reduction in fibre fractions with *Trichoderma* treatment, which may improve digestibility, while others observe

minimal changes depending on crop type and environmental conditions (Godlewska and Ciepiela, 2020; Akdag et al., 2024). This suggests that even though *Trichoderma* spp. supports overall plant health and metabolic function, their effectiveness on fibre composition is context-dependent.

Total sugars and carbohydrate content are also positively influenced by *Trichoderma*-based biostimulants. Increased accumulation of soluble sugars under drought stress is particularly important, as these compounds function as osmoprotectants, helping plants maintain cellular integrity (Mona et al., 2017). Enhanced carbohydrate metabolism has been linked to improved stress resilience and energy availability for growth and recovery.

Overall, the literature suggests that *Trichoderma* application can improve both yield and nutritional quality of forage crops under drought stress, although responses may vary with species, strain, and environmental conditions. There is a profound lack of targeted research investigating the effect of biostimulants specifically on alfalfa yield and quality under defined drought stress conditions. Furthermore, existing studies often treat plants as a uniform crop, neglecting the fundamental role of genetic variation. The potential for a genotype x biostimulant interaction on yield and quality parameters remains almost entirely unexplored in alfalfa productivity. It is plausible that a drought-sensitive alfalfa variety may exhibit a different magnitude of yield and quality response to a given biostimulant compared to a drought-tolerant variety. Therefore, the central research gap, and the focus of this thesis, is to quantitatively assess how their efficacy in safeguarding yield and quality is influenced by the genetic background of the alfalfa genotype under drought stress.

3. MATERIALS AND METHODS

3.1 Description of the experimental site

A three-year field experiment was conducted at the University of Debrecen, Institutes for Agricultural Research and Educational Farm, Botanical and Exhibiton Garden, Hungary, during the 2023, 2024 and 2025 cropping seasons. The experimental site has geographical coordinates positioning of 47°33'02'N; 21°35'56'E. The soil of the study area is homogeneous, which is classified as Calcic Endofluvic Chernozem according to the IUSS Working Group in 2014 in the World Reference Base for Soil. The soil chemical analysis revealed that the pH was considerably acidic at 0 –20 cm depth and moderately basic at 20 – 40 cm. Zn, Si, Na, and organic carbon (OC) were low, while N, P, and K were moderate (**Table 2**).

Table 2: Soil chemical properties of the experimental site

Parameters/Depth	0 – 20 (cm)	20 – 40 (cm)
pH (KCl)	6.93	7.46
OC (%)	2.89	2.87
P (mg/kg)	1538	1149
N (mg/kg)	82.2	53.4
K (mg/kg)	638	586
Na (mg/kg)	73.7	68.6
Si (mg/kg)	35.8	28.0
Zinc (mg/kg)	2.77	2.24

**KCl Potassium chloride soluble*

The average precipitation during the 2023 cropping season from January to September was 55.91 mm, slightly higher than the 30-year average of 47.1 mm, indicating 2023 was much wetter than normal. The 2024 average precipitation declined by 1.46 mm compared to the 30-year average, depicting slightly drier-than-normal conditions. Similarly, the average precipitation in the 2025 cropping season also declined by 8.28 mm compared to the 30-year average, demonstrating medium drier than normal (**Figure 2**). The average temperature ranges between 14.3 °C to 15.74 °C, indicating an average of 2.64 °C higher than 12.38 °C for a 30-year average temperature (**Figure 2**).

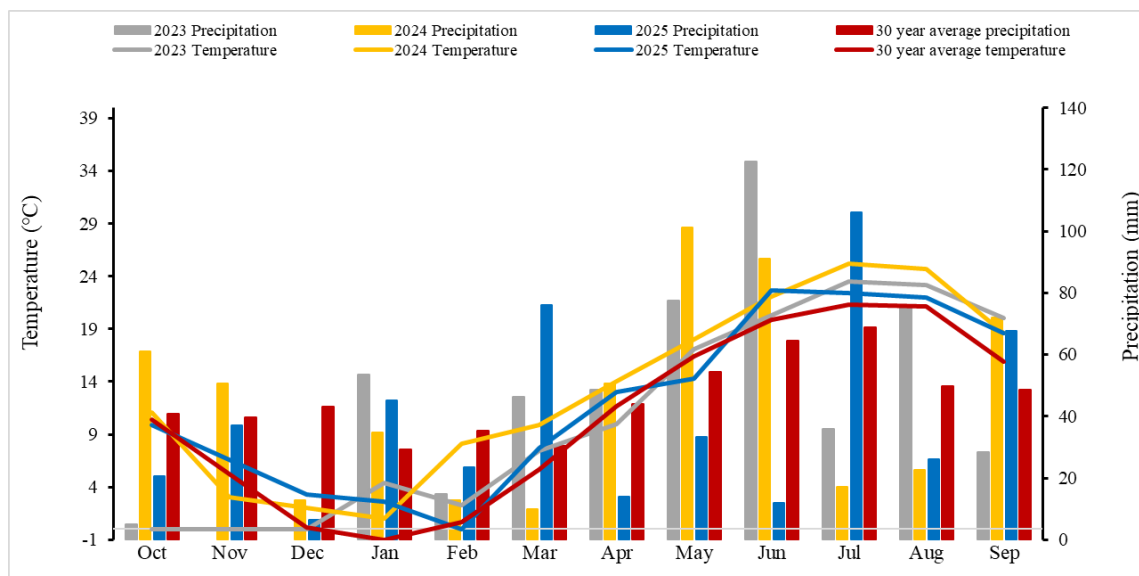


Figure 2: Meteorological data of the University of Debrecen experimental garden during the three-year study period (2023-2025) and the average for the past 30 years (Debrecen). Source: Data from the measurement program of Agrometeorological Observatory (AMO) of the Centre for Precision Farming R&D Service, University of Debrecen, Hungary

3.2 Experimental design and treatment

The alfalfa experiment was sown in March, 2022, after maize forecrop. The tested crop used was two different alfalfa genotypes, namely Magor variegated alfalfa (*Medicago x varia* Martyn) and Hunor-40 alfalfa (*Medicago sativa* L). These genotypes were obtained from the UD Institutes for Agricultural Research and Educational Farm Nyíregyháza Research Institute.

The experiment was arranged in a randomized, completely blocked design with four treatment levels replicated three times. A plot size of (3 x 4 m = 12 m²) was used with 1 m allays between plots and 2 m between replications. **Table 3** represents the treatment used.

Table 3: Treatment labels and levels used for the experiment

Code	Treatment	Dosage
T0	Control	-
T1	Biostimulant containing Si, MTU [®] PCA	2 L ha ⁻¹
T2	Tricho Immun + Ino Green	3 + 3 L ha ⁻¹
T3	Tricho Immun	3 L ha ⁻¹

* MTU[®]: 1-(2-methoxyethyl)-3-(1, 2, 3-thiadiazol-5-yl) urea plant phytohormone, *PCA: Pidolic acid; *Tricho Immun is the first internal Trichoderma. Composition: Patented strains of *Trichoderma afroharzianum* (TR04), *Trichoderma simmonsii* (TR05), 200 million (2x10⁸) CFU g⁻¹, *Ino Green is a liquid foliar fertilizer that contains calcium in calcite form and silicon. Composition: N (66 g L⁻¹), Ca (448 g L⁻¹), Si (56 g L⁻¹), Zn (24 g L⁻¹), Mn (8 g L⁻¹)

Tricho Immun was applied a day after cutting into the soil. In contrast, biostimulants containing MTU[®], pidolic acid, Si and Ino Green were also applied a week after regrowth to the leaves. No chemical fertilizer and pesticides were applied prior to the sowing and after the regrowth of the plants.

3.3 Seasonal harvesting schedules for alfalfa (*Medicago sativa* L.) and variegated alfalfa (*Medicago x varia* Martyn) forage production

Five separate harvests were made from the experiment during the 2023 to 2025 cropping season on the above various dates (**Table 4**). Prior to the first harvest, the plants under test received no treatment. Nonetheless, assessments and treatments were done following the initial harvest, and after the 5th harvest, no treatments or measurements were carried out.

Table 4: Harvesting period across growing season

Harvesting	1 st Cut	1 st Phase	2 nd Phase	3 rd Phase	4 th Phase
2023	May 22 nd	June 20 th	July 19 th	August 21 st	September 26 th
2024	April 29 th	June 13 th	July 10 th	August 12 th	September 20 th
2025	May 7 th	June 17 th	July 17 th	August 19 th	October 7 th

** 1st phase: from 1st to 2nd cutting; 2nd phase: from 2nd to 3rd cutting; 3rd phase: from 3rd to 4th cutting, 4th phase: from 4th to 5th cutting.

3.4 Data collection procedures and measurements

The data collection was conducted systematically using advanced instrumentation and well-structured sampling methods. The measurements included a broad spectrum, ranging from physio-morphological parameters, including plant height, and normalized difference vegetation index (NDVI). In addition, detailed assessments of dry matter yield, yield components and quality were performed, including nutritional composition, protein yield and photosynthetic traits essential to alfalfa productivity.

3.4.1 Plant height

To assess crop growth, plant height was measured as the distance from the soil surface to the apical meristem (apex) using a graduated metre rule. Measurements were taken on six randomly selected plants per plot to obtain a representative average. This method provides a simple yet reliable indicator of vegetative development and canopy expansion throughout the growing season. Plant height data were collected consistently at each growth stage to monitor variations in growth dynamics among treatments.

3.4.2 The normalised difference vegetation index (NDVI)

The Normalized Difference Vegetation Index (NDVI) was recorded weekly throughout the alfalfa growth cycle to monitor crop health and vigor. Measurements were taken using a Trimble Greenseeker NDVI meter, which calculates NDVI by analyzing red light (650 ± 10 nm) and near-infrared light (770 ± 15 nm), as outlined by Panek and Gozdowski, (2020) and described in equation (1). Reflectance data were obtained by positioning the instrument 1.0 m above the canopy and recording readings for each plot.

$$\text{NDVI} = (\text{NIR} - \text{R}) / (\text{NIR} + \text{R}) \dots\dots\dots (1)$$

Where R is the reflectance in the red range and NIR is the reflectance in the near-infrared range

3.4.3 Net photosynthetic rate

Net photosynthetic rate was measured on intact leaves using a LI-6800 portable photosynthesis system (LI-COR, Lincoln, NE, USA). The system is equipped with two high-precision infrared gas analyzers that determine CO₂ and H₂O mole fractions in air. Based on input and output values of CO₂ (mol mol⁻¹) and H₂O (mmol mol⁻¹), along with leaf temperature (°C), atmospheric pressure (kPa), flow rate (mol s⁻¹), and other parameters, the instrument calculates net assimilation, transpiration, total conductance to CO₂, and intercellular CO₂ concentration, following VonCaemmerer and Farquhar (1981). Light in the sample chamber was maintained at 1500 μmol photons m⁻² s⁻¹ PAR, with 90% red (625 nm) and 10% blue (475 nm) provided by the LI-6800-01A chamber head (2 cm² aperture). Chamber CO₂ concentration was controlled at 400 μmol mol⁻¹ using an injector and CO₂ cartridges, while the average ambient CO₂ level was 399.984 μmol mol⁻¹. Leaf temperature was monitored by two thermocouples in contact with the lower leaf surface (LI-COR, Inc (2017)). Measurements were conducted on light-adapted leaves, with six readings per leaf on three plants per plot. Data were logged once parameters stabilized, after a minimum of two minutes LI-COR, Inc (2017). Air temperature inside the chamber was also recorded by the LI-6800.

3.4.4 Biomass yield measurement and moisture standardisation

Fresh biomass yield was determined by harvesting all plants within each plot using a mowing device. The harvested biomass was collected, bulked, and immediately weighed with a digital scale to obtain plot-level total fresh weight (kg m⁻²). These fresh weights were then multiplied by 10,000 to convert from kg m⁻² to kg ha⁻¹. From each plot, a representative subsample was collected and weighed to determine fresh weight in grams (g). The subsamples were then oven-dried at 105°C for 72 hours and reweighed for dry

weight (g) to facilitate the calculation of dry matter (DM %) using equation 1 (Hidosa and Kibret 2021).

$$\text{Dry matter (DM \%)} = \frac{\text{Dry weight (g)}}{\text{Fresh weight (g)}} \times 100 \dots\dots\dots (2)$$

The harvested fresh biomass yield per plot was then converted to dry matter yield using the measured DM percentage, as this provides the true crop dry matter yield per plot.

$$\text{Dry matter yield (kg ha}^{-1}\text{)} = \text{Fresh biomass yield (kg ha}^{-1}\text{)} \times \text{Dry matter (DM \%)} \dots\dots\dots (3)$$

Furthermore, to be able to do a comparison across harvests and treatments, dry matter yields of alfalfa were standardised to 15% moisture (85% DM) using equation 3. This moisture level falls within the widely recommended safe baling range for alfalfa hay, typically 15 – 20% moisture, which minimises leaf loss and prevents spoilage during storage and also preserves quality and reduces dry matter losses (Wilcke et al., 1999; Johnson, 2020).

$$\text{Standardised Yield (15\% moisture)} = \frac{\text{Dry matter yield (kg DM ha}^{-1}\text{)}}{0.85} \dots\dots\dots (4)$$

3.4.5 Determination of protein yield

Protein yield was calculated using the formula 'protein yield (kg ha⁻¹) = CP x DMY .(5) (Atumo et al., 2021), where CP = crude protein content in percentage and DMY = dry matter yield in kg ha⁻¹

3.4.6 Quality traits

Plant samples from each plot were taken at harvest during the 2023, 2024 and 2025 cropping seasons. These samples were submitted to the laboratory of the University of Debrecen for the determination of dry matter content and quality parameters. 500 g of fresh biomass sample was weighed and dried at 103°C in an oven until a constant weight to determine dry matter content. Dry samples were grounded to pass through a 1 mm mesh sieve and analysed for nitrogen using the Kjeldahl method and fibre content. Protein content was calculated as N x 6.25. Total sugars and total carbohydrates were determined according to (Thomas and Dutcher, 1924). The fat content of the samples was determined by the Randall modification of the Soxhlet method (AOAC 2003). Fat content (m/m) % = ((M 2 – M 1)/ (M 3 - M 1))*100. Where M 1: weight of the extraction crucible (g). M 2 = weight of the extraction crucible + dried petroleum ether extract (g), M 3 = weight of the extraction crucible + weight of the test sample (g). Lastly, saturated fatty acid analysis was performed, where the total lipid extracted sample was mixed with hexane and NaOH:MeOH, then heated at 80°C for 10 min. After saponification, water is added, and unsaponifiable matter is extracted with hexane (waste). The aqueous layer is acidified

with H₂SO₄; fatty acids are extracted with hexane and reacted with BF₃:MeOH at 80°C for 30 min. Finally, NaCl is added to salt out the product, and the hexane phase is dried with sodium sulfate and analysed by gas chromatography.

3.5 Assessment of drought stress conditions via the Pálfai drought index (PaDI)

The Pálfai Drought Index (PaDI) was employed to characterize the intensity and temporal variability of water scarcity during the study period. This index integrates key climatic parameters to provide a quantitative measure of drought conditions. In this study, the modified version of the Pálfai Drought Index proposed by Pálfai and Herceg (2012) was applied. The computation begins with the determination of a base value, which serves as the foundation for incorporating precipitation and temperature components in subsequent steps using the formula below.

$$PaDI_0 = \frac{\left[\sum_{i=apr}^{aug} T_i \right] * 100}{5 \left(c + \sum_{i=oct}^{sept} (P_i * w_i) \right)}$$

PaDI₀ = base-value of drought index (°C 100 mm⁻¹)

T_i = monthly mean temperature from April to August (°C)

P_i = monthly sum of precipitation from October to September (mm)

w_i = weighting factor (**Table 5**)

c = constant value (10 mm)

Table 5: Monthly Weighting Factor Values (w_i) for Computing the Pálfai Drought Index (PaDI)

Month	Wi
October	0.1
November-December	0.4
January-April	0.5
May	0.8
June	1.2
July	1.6
August	0.9
September	0.1

Calculation of drought index correction factors (k₁, k₂, k₃)

k₁ = temperature correction factor

$$k_1 = \frac{(T_{jun} + T_{jul} + T_{aug})/3}{(\bar{T}_{jun} + \bar{T}_{jul} + \bar{T}_{aug})/3}$$

$T_{jun} + T_{jul} + T_{aug}$ = annual mean temperature for June-August (°C)

$\bar{T}_{jun}, \bar{T}_{jul}, \bar{T}_{aug}$ = 30-years mean temperature for June-August for period 1991-2020 (°C)

k_2 = precipitation correction factor

$$k_2 = \sqrt[4]{\frac{2 * \bar{P}_{summer}^{min}}{MIN(P_{jun}, P_{jul}, P_{aug}) + \bar{P}_{summer}^{min}}}$$

$\bar{P}_{summer}^{min}$ = the lowest value from 30-years precipitation sum of three summer months (June, July, August) (mm)

$MIN(P_{jun}, P_{jul}, P_{aug})$ = the lowest value from annual precipitation sum of three summer months (June, July, August) (mm)

k_3 = correction factor, which characterizes the precipitation circumstances of the previous period

$$k_3 = \sqrt[n]{\frac{\bar{P}}{\bar{P}_{36m}}}$$

n = reduction factor, value is 3.0 on the plain area, on hilly or higher territories is 5.0

$\bar{P}$ = average multi-annual precipitation sum for period October-September (mm)

$\bar{P}_{36m}$ = average precipitation sum for period October-September for previous three years (mm)

$$\bar{P}_{36m} = \sum_{i=(year-3)Oct}^{(year)Sept} P_i / 36$$

P_i = monthly precipitation sum (mm)

Calculation of Palfai Drought Index (PaDI) (°C 100 mm⁻¹)

$$PaDI = PaDI_0 \times k_1 \times k_2 \times k_3$$

3.6 Classification of drought severity across the experimental years

The modified Pálfai Drought Index constitutes a robust metric for evaluating drought severity via a unified numerical value applicable to varied agricultural seasons. Derived from hydrological years (October–September) and integrates temperature records with weighted monthly precipitation aggregates. As drought severity is shaped not merely by current-year precipitation but also antecedent conditions, precipitation from preceding years (36 months) is integrated as a corrective adjustment. Its principal strength lies in

facile computation from accessible meteorological datasets, furnishing a dependable gauge of drought magnitude and its potential impact for crop productivity.

Nevertheless, per the PaDI computations, 2023 registered mild drought ($\text{PaDI} = 4.3 \text{ }^{\circ}\text{C } 100 \text{ mm}^{-1}$), 2024 moderate drought ($\text{PaDI} = 6.5 \text{ }^{\circ}\text{C } 100 \text{ mm}^{-1}$), and the 2025 growing season medium drought ($\text{PaDI} = 9.4 \text{ }^{\circ}\text{C } 100 \text{ mm}^{-1}$) (**Table 6**).

Table 6: The calculated Palfai Drought Index during three growing seasons in the experimental site in Debrecen (2023-2025).

Year	$P_{36 \text{ month}}$ mm	Monthly average P for previous 36 month mm	Sum of precipitation April-August mm	$\text{MIN}(P_{\text{jun}}, P_{\text{jul}}, P_{\text{aug}})$ mm	PaDI_0 $^{\circ}\text{C } 100 \text{ mm}^{-1}$	PaDI $^{\circ}\text{C } 100 \text{ mm}^{-1}$	Drought category
2023	1567.3	43.5	359.8	35.9	3.76	4.3	mild drought
2024	1761.7	48.9	282.2	17.0	5.19	6.5	moderate drought
2025	1823.0	50.6	191.9	17.9	8.21	9.4	medium drought

Note: $P_{36 \text{ month}}$ = precipitation sum for period October-September for previous three years; $\text{MIN}(P_{\text{jun}}, P_{\text{jul}}, P_{\text{aug}})$ - the lowest precipitation from the sum of summer months: June, July, August.

3.7 Statistical analysis

The factors (biostimulants and alfalfagenotype) determining abiotic stress tolerance and their impacts on productivity and quality under contrasting drought stress were assessed through the analysis of variance (ANOVA) using the Genstat statistical software package, 18th edition. Prior to the analysis, the data gathered was passed through a normality test using the Shapiro-Wilk test, with most parameters showing a normal distribution. Mean values were separated at least significantly different (LSD) at a 5% probability level. Duncan's multiple range test (DMRT) was performed to compare and rank the individual treatment and various interactions performance from highest to lowest, as illustrated in the accompanying tables and graphs. Pearson correlation and regression analyses were carried out to explore the strength and direction of relationships among the measured traits, helping to identify which variables were most closely associated. These analyses are particularly useful for detecting linear associations between physiological,

morphological, and yield-related traits, which can provide early indicators of crop performance.

In addition, a principal component analysis (PCA) was performed using R statistical software (Soren, 2006) to investigate the interrelationships among all measured parameters while reducing data complexity and retaining the most meaningful variation. PCA was applied to identify correlations and patterns among key factors influencing alfalfa performance, including biostimulant levels, seasonal variability, and genotype differences, in relation to morpho-physiological parameters. This multivariate approach is important because it highlights underlying trait groupings, reveals how multiple factors interact simultaneously, and helps to pinpoint which variables contribute most strongly to growth and productivity. Collectively, these statistical tools provide a deeper understanding of the interactions among traits and their combined impact on alfalfa performance, supporting more informed interpretations and management decisions.

4. RESULTS AND DISCUSSIONS

4.1 Evaluation of the effects of biostimulants, genotype variability and environment on the growth and physiology of *Medicago sativa* L. and *Medicago x varia* Martyn under contrasting drought stress

4.1.1 Differential effects of biostimulant treatments on plant height in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023-2025)

Morphological responses of two distinct alfalfa genotypes to various biostimulant treatments were evaluated during the 2023, 2024, and 2025 cropping seasons under contrasting drought stress conditions. The findings revealed differential responses in alfalfa plant height to biostimulant treatments across all four phases (**Figure 3**).

In the 2023 cropping season (mild drought stress), biostimulant treatment application showed no statistically significant ($P > 0.05$) impact on plant height at 14 days after harvest (DAH) during the first phase. However, highly significant ($P > 0.001$) differences emerged at 21 and 28 DAH. Amongst the treatments applied, T2 consistently yielded the highest plant heights, recording 58.1 cm and 72.3 cm, reflecting 29.8% and 12.8% increases over the control, respectively. A comparable delayed efficacy was observed in the second phase, with T2 again producing the tallest plants, indicating 36.6% and 14.2% increases over the control at 21 and 28 DAH. In the third phase, the effects of these treatments were less pronounced, with no significant differences at any stage. However, numerically, plant heights at 14 DAH range from 31.5 to 34.1 cm and 60.2 to 64.3 cm at 28 DAH. In the fourth phase, a significant difference was only observed at 28 DAH, where T1 and T2 achieved maximum heights of 74.7 cm and 74.4 cm, respectively.

During the 2024 cropping season (moderate drought stress), a consistent temporal pattern emerged across all phases: non-significant effects at 14 DAH but highly significant differences at 21 and 28 DAH. In the first phase, T2 resulted in the greatest heights of 60.4 cm and 77.4 cm, reflecting a 12.5% and 15.9% increase in height over the control. This delayed effectiveness persisted in the second phase, with T2 increase height by 15.3% and 13.9% over untreated plots (T0). In the third and fourth phases, a similar pattern was observed. Plant height showed no significant differences among treatments at 14 DAH but significant variations by 21 and 28 DAH. Among the treatments applied, T2 consistently produced the highest plant heights, measuring 56.7 cm and 71.1 cm in Phase 3, and 59.6 cm and 73.1 cm in Phase 4. These values represent height increases over the control of 12.5% and 18.7% in Phase 3, and 17.3% and 12.9% in Phase 4, underscoring T2's enhanced growth-promoting effect during later developmental stages.

In the 2025 cropping season (medium drought stress), biostimulant treatment application showed no statistically significant ($P > 0.05$) impact on plant height at 14 days after harvest (DAH) during the first phase. However, highly significant ($P > 0.001$) differences emerged at 21 and 28 DAH. Amongst the treatments applied, T2 consistently yielded the highest plant heights, recording 56.2 cm and 83.9 cm, reflecting 12.6% and 13.5% increases over the control, respectively. This consistent pattern persisted into the 2nd phase; no significant treatment differences were observed at 14 DAH, but they became evident at 21 and 28 DAH, respectively. T2 again produced the tallest plants, measuring 61.5 cm and 77.3 cm at 21 and 28 DAH, respectively, representing height increases of 17.4% and 15.9%. In the third phase, effects of these treatments were less pronounced, with no significant differences at 14 DAH, but biostimulants significantly affected height at 21 and 28 DAH. T2 treatment continued to lead, with heights of 69.4 cm and 82.8 cm at 21 and 28 DAH. T1 and T3 demonstrated intermediate heights, while T0 remained the lowest. In the 4th phase, biostimulant effects remained non-significant at 14 DAH but demonstrated significant differences at 21 and 28 DAH. T2 consistently yielded the greatest plant heights, measuring 53.9 cm and 66.7 cm, while T0 exhibited the lowest growth, representing increases of 24.8% and 21.3% compared to the control groups.

Across the 2023–2025 cropping seasons under four sequential cutting phases, our study demonstrated that application of the biostimulant treatments consistently enhanced alfalfa plant height under mild to moderate drought stress, with effectiveness becoming evident approximately three weeks after treatment application. A consistent non-significant observed during early responses at 14 DAH suggests that the applied biostimulant treatments in alfalfa plants need ample time to activate physiological processes such as root growth stimulation, nutrient uptake, and hormonal signaling, rather than inducing immediate morphological changes (Mouradi et al., 2012; Sosnowski, 2018; Appiah et al., 2024a). The observed consistently superior performance of T2 by increasing height ranged from 12% to 37% under mild to moderate drought stress across all seasons, indicating the strong capacity of T2 to improve drought tolerance and growth efficiency in alfalfa plants. These findings align with previous studies reporting significant increases in growth traits by 68.1% under the application of *Trichoderma* spp. over the control plots (Zhang et al., 2019b). This improvement may be attributed to enhanced water-use efficiency, improved cell expansion, and activation of stress-responsive metabolic pathways, which collectively support sustained shoot elongation even under moisture deficit conditions (Li et al., 2022; Prince et al., 2022). Similar studies linked growth

improvements to biostimulants, which enhance soil nutrient availability and enzyme activity and improve physiological processes like osmotic regulation, antioxidant capacity, and photosynthesis under stress (Zhang et al., 2019b; Wang et al., 2024; Akdağ et al., 2024). Such enhancements lead to improved overall growth, as observed in studies where humic acid and protein hydrolysate act as plant growth regulators by promoting nitrogen absorption and metabolism, indirectly affecting seedling growth parameters and resilience (Akdağ et al., 2024; Sun et al., 2024).

Additionally, a remarkable pattern observed throughout the study was the differential responsiveness to biostimulants across successive cutting phases, with early regrowth stages exhibiting more pronounced increases in plant height relative to later cuttings, particularly evident during extended drought conditions in 2023. This disparity suggests that younger or actively regenerating tissues are more amenable to biostimulant-mediated physiological enhancements, whereas accumulating drought stress progressively constrains plant plasticity and adaptive responsiveness.

Moreover, escalating drought severity from mild to moderate levels did not impair the efficacy of biostimulants. On the contrary, relative growth increments were frequently greater under intensified stress conditions, as particularly evident in the 2025 cropping season, underscoring that these biostimulant treatments primarily function as enhancers of stress resilience rather than mere growth promoters. Many studies suggest that under water deficit, treated plants likely maintained better cellular hydration, osmotic adjustment, and metabolic activity, enabling continued elongation compared with untreated controls (Baltazar et al., 2021; Hou et al., 2022). Furthermore, the uniform delayed response observed across all cropping years underscores the critical importance of application timing in biostimulant management strategies. The maximum benefits were attained 21–28 days post-application, indicating that these products should be deployed proactively rather than as immediate remedial interventions during drought stress conditions.

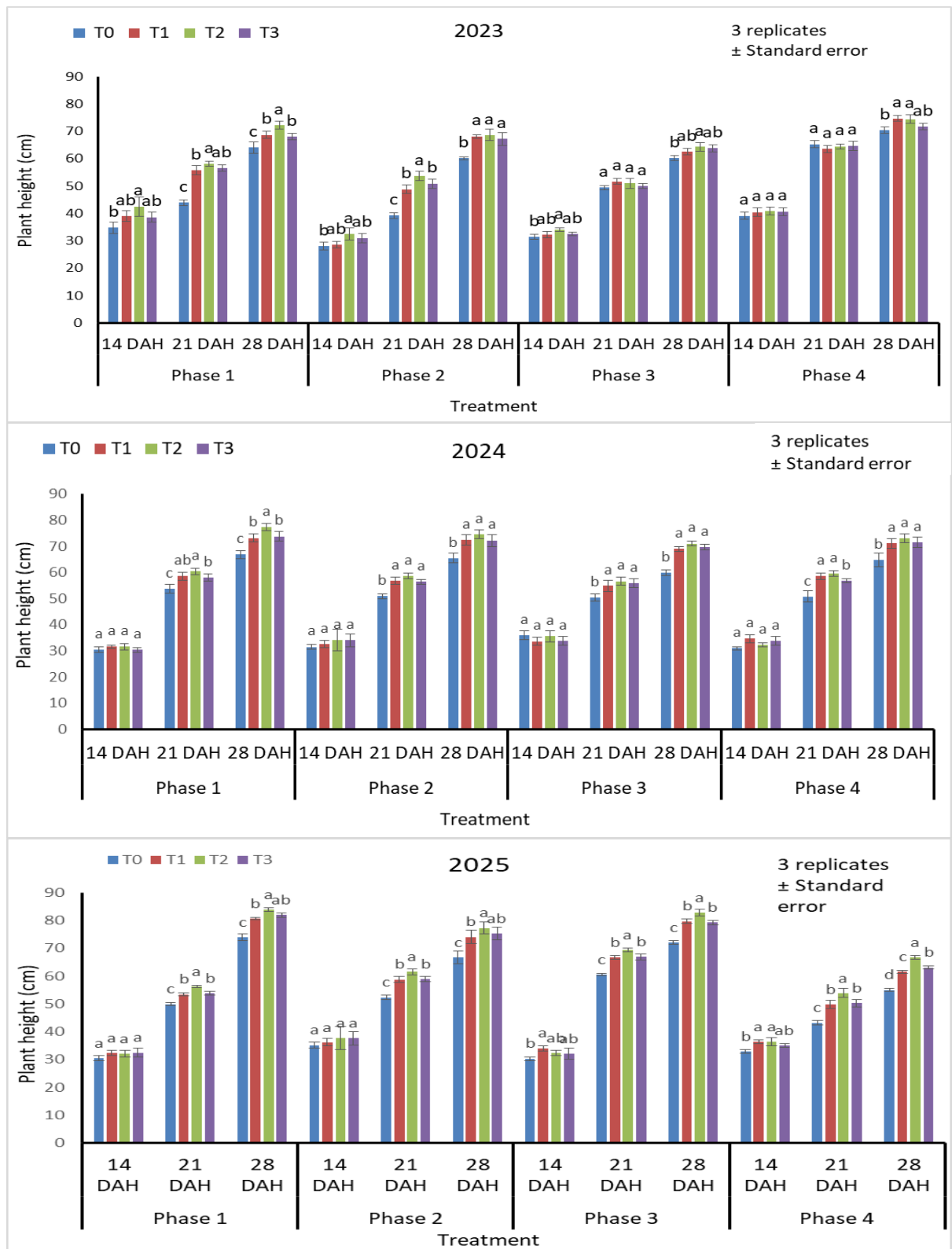


Figure 3: Effect of biostimulant treatments on plant height (cm) of alfalfa crop during the three cropping seasons (Debrecen, 2023–2025).

Note: Columns represent annual mean values of genotypes (2) and repetitions (3) $\pm$ standard error ($n = 6$). Different letters mean significant differences between treatments at the same phase at $p = 5\%$. ** 1st phase: from 1st to 2nd cutting; 2nd phase: from 2nd to 3rd cutting; 3rd phase: from 3rd to 4th cutting, 4th phase: from 4th to 5th cutting.

4.1.2 Differential effects of genotypic variation on plant height in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023-2025)

The analysis of variance reveals that plant height exhibited significant variability across genotypes throughout all four experimental phases (**Figure 4**). However, genotypic (G) variation contributed to disparities in plant height, although its significance fluctuated across phases. During Phase 1 of the 2023 (mild drought stress) cropping season, Magor consistently exhibited superior performance compared to Hunor across all measured time points, with statistically significant differences observed at 14, 21 and 28 DAH. By 28 DAH, Magor attained a height of 71.3 cm, representing a 9.2% increase in growth relative to Hunor, which measured 65.3 cm. Phase 2 revealed comparable trends, with Magor consistently achieving significantly greater plant heights at all developmental stages. Conversely, Phase 3 demonstrated a diminished genotypic influence, as statistically significant differences were only apparent at 21 DAH, with no significant variation observed at either 14 or 28 DAH. Similarly, genotypic effects in Phase 4 remained largely diminished, with no significant disparities at 14 or 21 DAH; however, a statistically significant difference emerged at 28 DAH, where Magor achieved 74.3 cm compared to Hunor's 71.4 cm, reflecting a 4.1% increase relative to Hunor. Collectively, Magor generally exhibited a tendency for producing taller plants under mild drought stress conditions, though the consistency of this effect diminished in subsequent developmental phases.

In the 2024 cropping season (moderate drought stress), genotypic variation influenced plant height, though its significance varied. In Phase 1, the Magor genotype consistently exceeded Hunor in height at all growth stages, with statistically significant differences at 14, 21 and 28 DAH ($P < 0.05$ and $P < 0.001$), respectively. Progression from 21 DAH to 28 DAH: Magor reached 75.9 cm, an 8.9% increase over Hunor. Phase 2 exhibited similar trends, with Magor again producing significantly taller plants at all stages ($P < 0.05$). Across Phases 3 and 4, Magor maintained a consistent genotypic advantage at 14, 21 and 28 DAH, achieving 74.1 cm compared to Hunor's 66.1 cm, reflecting a 12% increase in height relative to Hunor. Overall, Magor generally demonstrated superior plant height under moderate stress conditions, showing a consistent growth increase exceeding 10% across all experimental phases.

In the 2025 cropping season (medium drought stress), alfalfa genotypes demonstrated differential height performance across the experimental phases. In the 1st Phase, genotypic variations were not statistically significant at 14 DAH; however, significant

differences emerged at 21 and 28 DAH, with the Magor genotype consistently exhibiting superior height values during these later stages. During the 2nd Phase, genotypic differences proved significant at all recorded stages, with the Magor genotype consistently surpassing the Hunor genotype in height throughout this phase, achieving 59.4 cm at 21 DAH and 78.1 cm at 28 DAH. In the 3rd Phase, genotype-specific effects on height remained significant across all growth stages, with Magor continuing to display enhanced growth compared to Hunor, particularly evident in the largest differences observed at 28 DAH. For the 4th Phase, no significant ($P > 0.05$) genotypic difference was detected at 14 DAH. Nevertheless, significant differences reappeared at 21 and 28 DAH, where Magor genotype again produced greater plant heights. Overall, Magor consistently outperformed Hunor under medium drought stress conditions, especially beyond the initial stages of each experimental phase. Furthermore, the lack of significant interaction across all the cropping seasons (2023-2025) generally indicated a uniform response to treatments.

However, across all the cropping seasons under varying drought stress conditions, plant height exhibited significant genotypic variation between alfalfa genotypes (Magor and Hunor) across all harvesting phases, with Magor consistently outperforming Hunor. This superiority was evident particularly in moderate (2024 season) and medium (2025 season) drought stress, where differences proved more stable and pronounced across phases. These findings align with prior research which reported substantial genotypic variability in growth performance under water deficits (Luo et al., 2020; Bhattacharya, 2021a; Nour et al., 2024; Hou et al., 2022; Prince et al., 2022). Numerous studies shows that drought stress reduces shoot growth and plant height, though the magnitude varies by genotype (Farooq et al., 2008; Prince et al., 2022). Drought-tolerant cultivars typically sustain greater height and biomass accumulation than sensitive ones (Khodayari and Abedini, 2022); however, Magor performance from our studies reflects an inherent genetic advantage enabling improved growth maintenance under stress conditions. A key observation was that genotypic differences grew more consistent and pronounced due to changes in drought stress severity: under mild stress, effects were present but less stable across phases, whereas under moderate and medium stress, Magor showed height increases exceeding 10% relative to Hunor across many phases, which demonstrated its superior performance over Hunor across these stress conditions. This trend corroborates reports that drought amplifies genotypic differentiation in both annual and perennial legumes (Su et al., 2019; Wang et al., 2023). Similar studies highlight that a genotype's

superior performance is often contingent on the specific type and timing of drought stress (terminal vs. intermittent), making it difficult to breed universally resilient varieties (Iseki et al., 2018; Gonçalves et al., 2019; Putnam et al., 2021). In low-stress conditions, phenotypic plasticity often masks genetic differences, but intensifying stress highlights adaptive traits such as enhanced water-use efficiency, osmotic adjustment, and root architecture. Similar trends appear in alfalfa breeding studies, where tolerant cultivars express adaptive potential more clearly under moderate to severe water deficit conditions (Erice et al., 2009; Hou et al., 2022). Notably, genotypic differences were often non-significant at 14 DAH, but significance emerged at 21 and 28 DAH, suggesting progressive activation of drought-tolerance mechanisms beyond early establishment. These findings align with previous studies indicating that initial vegetative growth relies mainly on seed reserves and vigour, while later stages depend on physiological regulation and stress adaptations (Hou et al., 2022; Luo et al., 2020). Drought-tolerant genotypes often sustain cell expansion, delay growth inhibition and maintain photosynthetic activity under prolonged stress trends consistent with the Magor later-stage dominance observed in this study. Shoot growth maintenance under drought typically involves complementary physiological and morphological adaptations (Valluru et al., 2026; Kaciene et al., 2022), including enhanced root development, deeper rooting, improved water-use efficiency, greater antioxidant activity, membrane stability and osmotic adjustment in tolerant alfalfa genotypes (Prince et al., 2022; Chen et al., 2024). Magor consistent height advantage thus likely stems from improved root and physiological traits. Moreover, the absence of significant genotype x biostimulant interactions further highlights Magor's intrinsic genetic resilience over treatment-specific responses. Overall, our study demonstrated enhanced alfalfa growth under varying drought stress conditions while highlighting the genetic stability of Magor's superiority over Hunor across varying drought intensities. These findings provide valuable insight for genotype selection and breeding strategies aimed at improving drought resilience in alfalfa production systems.

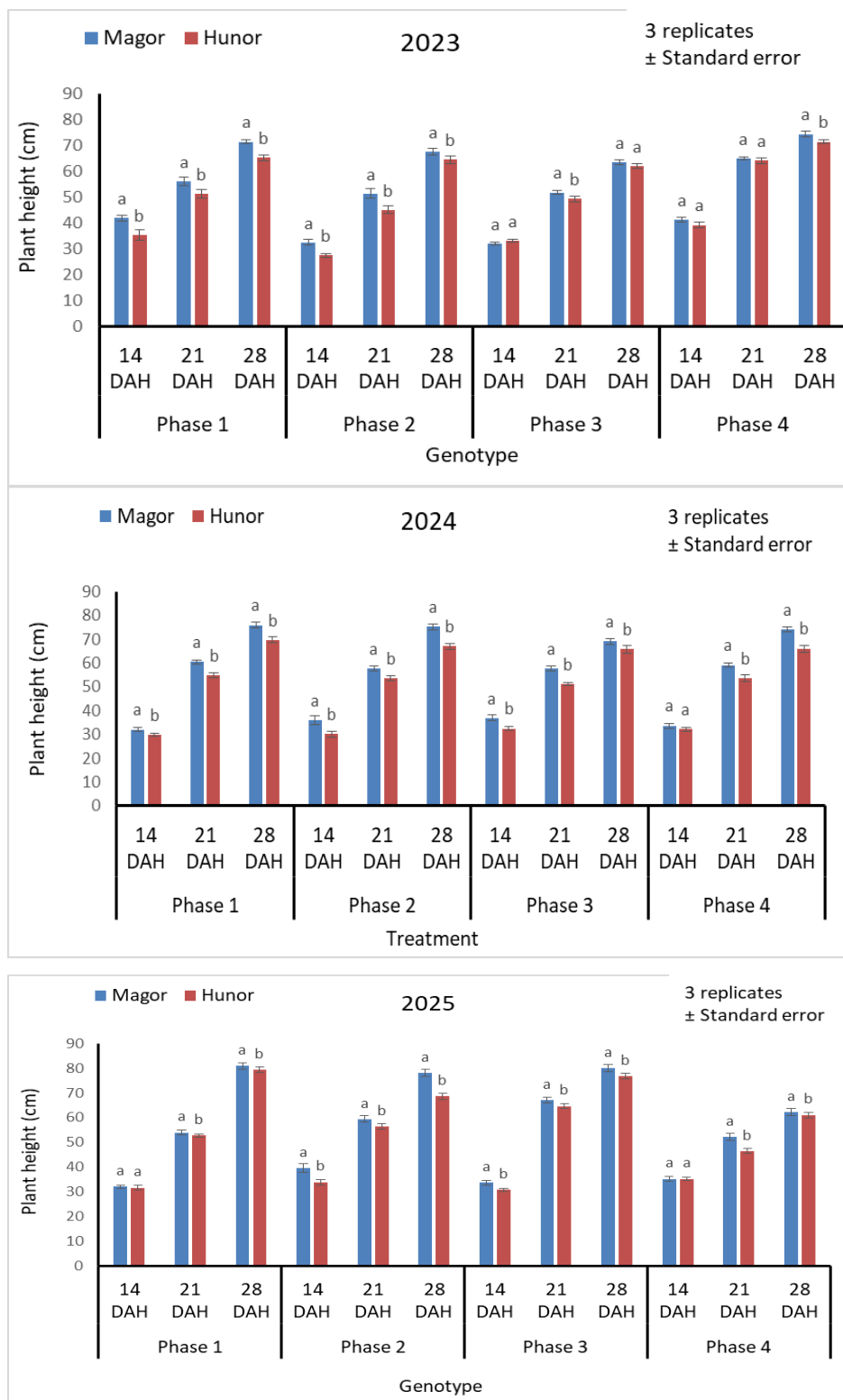


Figure 4: Effect of genotypic variation on plant height (cm) of alfalfa crop during the three cropping seasons (Debrecen, 2023–2025).

*Note: Columns represent annual mean values of treatments (4) and repetitions (3) ± standard error (n = 12). Different letters mean significant differences between genotype at the same phase at p = 5%. ** 1st phase: from 1st to 2nd cutting; 2nd phase: from 2nd to 3rd cutting; 3rd phase: from 3rd to 4th cutting, 4th phase: from 4th to 5th cutting.*

4.1.3 Interactive effects of biostimulant, genotype, and season on plant height of alfalfa across growth phases under contrasting drought conditions (2023–2025)

We analysed the interactive effects of biostimulant application, genotype and crop year on plant height of alfalfa at 14, 21, and 28 days after harvest (DAH) across four growth phases under contrasting drought stress conditions (**Table 7**).

In Phase 1, plant height was significantly influenced by genotype and season, but not by treatment. A significant genotype x season interaction indicated varying genotype performance across seasons. Specifically, under mild drought, Magor increased plant height by 18.7%, 7.4% under moderate drought, and 1.7% under medium drought compared to Hunor. All other interactions, including the three-way interaction, were non-significant at 14 DAH. At 21 DAH, treatment, genotype, and season significantly affected plant height, as did the treatment x season interaction, indicating that seasonal conditions influenced biostimulant effectiveness during mid-regrowth. Treatments T1, T2, and T3 were statistically similar, but T2 improved alfalfa height by 32% under mild drought, 12.4% under moderate drought, and 13% under medium drought compared to the control. The genotype x season interaction was also significant, with Magor outperforming Hunor by 9.4% in 2023 under mild drought, 10% in 2024 under moderate drought, and 2.2% in 2025 under medium drought. All other interactions, including the three-way interaction, were non-significant. At 28 DAH, genotype, treatment, and season significantly influenced plant height, along with a genotype x season interaction. Magor produced 9.3% higher height in 2023 under mild drought, 8.8% in 2024 under moderate drought, and 2.1% in 2025 under medium drought compared to Hunor. All other interactions, including the three-way interaction, were non-significant. Overall, Phase 1 results show that seasonal variability and genotypic differences primarily drove plant height variation, with biostimulant effects emerging mainly during mid- to late regrowth (21 and 28 DAH). The lack of three-way interactions at 14, 21, or 28 DAH indicates stable combined responses of genotype and biostimulant across seasons.

In Phase 2, at 14 DAH, plant height was significantly influenced by genotype and season, but not by treatment. No significant treatment x season, genotype x season, or three-way interactions were observed. However, the treatment x genotype interaction was significant, indicating differential genotype responses to biostimulant during early regrowth. Specifically, T2 increased height in Magor by 23.2%, followed by T3 (16.7%) and T1 (4.7%), whereas height in Hunor declined under T2 compared to the control. At 21 DAH, treatment, genotype, and season significantly affected plant height, as did the

treatment x season interaction, with T1, T2, and T3 statistically similar, but T2 improving height by 36% under mild drought, 15.1% under moderate drought, and 17.4% under medium drought compared to the control. The genotype x season interaction was significant, with Magor outperforming Hunor by 13.8% in 2023 under mild drought, 7.5% in 2024 under moderate drought, and 5.3% in 2025 under medium drought. All other interactions, including the three-way interaction, were non-significant. At 28 DAH, genotype, treatment, and season significantly influenced plant height, with a genotype x season interaction where Magor produced 4.7% higher height in 2023 under mild drought, 12.1% in 2024 under moderate drought, and 13.8% in 2025 under medium drought compared to Hunor. All other interactions, including the three-way interaction, were non-significant.

In Phase 3, at 14 DAH, plant height was significantly influenced by genotype and season, but not by treatment. A significant genotype x season interaction showed Magor producing 13.4% higher height in 2024 under moderate drought and 9.7% in 2025 under medium drought compared to Hunor; however, Hunor slightly outperformed Magor by 3.8% in 2023 under mild drought. All other interactions, including the three-way interaction, were non-significant. At 21 DAH, treatment, genotype, and season significantly affected plant height, as did the treatment x season interaction. Treatments T1, T2, and T3 were statistically similar, but T1 and T2 each improved height by 4.6% under mild drought; T2 enhanced height by 12.4% under moderate drought and 14.5% under medium drought compared to the control. The genotype x season interaction was significant, with Magor outperforming Hunor by 5.2% in 2023 under mild drought, 12.7% in 2024 under moderate drought, and 3.9% in 2025 under medium drought. All other interactions, including the three-way interaction, were non-significant. At 28 DAH, treatment, genotype, and season significantly affected plant height, as did the treatment x season interaction. Treatments T1, T2, and T3 were statistically similar, but T2 improved height by 6.7% under mild drought, 18.7% under moderate drought, and 15% under medium drought compared to the control. All other interactions, including the three-way interaction, were non-significant.

In Phase 4, at 14 DAH, plant height was significantly influenced by treatment and season, but not by genotype. No two- or three-way interactions were significant. At 21 DAH, treatment, genotype, and season significantly affected plant height, as did treatment x season and genotype x season interactions; the three-way interaction (treatment x genotype x season) was also significant. Numerically, in 2023 under mild drought, all

treatments were statistically similar, but T3 increased height in Magor by 3.9%, whereas Hunor height declined by 4 - 8% under treatments compared to the control. Under moderate drought in 2024, T1 and T2 increased height in Magor by 11%, and T2 increased height in Hunor by 23.8% compared to the control. Under medium drought in 2025, T2 increased height in Magor by 28.3% and in Hunor by 20.6% compared to the control, though individual treatments were statistically similar. At 28 DAH, treatment, genotype, and season significantly affected plant height, as did the treatment x season interaction. Treatments T1, T2, and T3 were statistically similar, but T1 and T2 each improved height by 5.9% under mild drought; T2 enhanced height by 12.9% under moderate drought and 21.3% under medium drought compared to the control. The genotype x season interaction was significant, with Magor outperforming Hunor by 4.1% in 2023 under mild drought, 12.1% in 2024 under moderate drought, and 1.7% in 2025 under medium drought. All other interactions, including the three-way interaction, were non-significant.

Overall, across the 2023–2025 cropping seasons, plant height of alfalfa was predominantly governed by genotype and seasonal drought intensity, with biostimulant effects becoming more pronounced during mid- to late-regrowth stages (21–28 DAH). Our results demonstrated that the three-way interaction among biostimulant application (B), genotype (G), and season/year (Y) was largely non-significant across growth phases and days after harvest, except at 21 DAH in Phase 4, where it significantly affected plant height. This indicates that genotype-specific responses to biostimulants were relatively stable across contrasting drought seasons. Similar results have been reported by Tamburino et al. (2025). In practical terms, the response pattern of each genotype to biostimulant treatment did not substantially shift from one year to another, despite variability in drought intensity between 2023 (mild drought) and 2025 (medium drought). However, the observed significant B x G x Y interaction at 21 DAH in Phase 4, where, under moderate drought in 2024, T2 markedly increased plant height in Hunor by 23.8% and in Magor by 11%, while under medium drought in 2025, T2 increased height in Magor by 28.3% and Hunor by 20.6% compared to the control suggests that under prolonged or cumulative drought exposure, more complex physiological adjustments may emerge, leading to season-dependent genotype responses at specific developmental stages. These findings align with studies on complex multi-factor interactions under severe stress in forage crops, where prolonged drought alters carbon allocation, hormonal balance, and treatment responsiveness (He et al., 2022; Kačienė et al., 2022). This stage-

specific interaction pattern is consistent with findings that plant responses to biostimulants are influenced by both environmental stress intensity and phenological stage (Baltazar et al., 2021; Akdağ et al., 2024). In addition, the minimal three-way interaction also implies that although seasonal variability strongly affected plant height, it did not fundamentally alter genotype-specific responsiveness to biostimulants in most cases. This stability is agronomically advantageous, as it suggests predictable performance of treatments across growing seasons. On the other hand, the observed significant genotype x season interactions at Phases 1–3 indicate that genotype performance varied depending on drought severity: Magor showed superior performance under moderate and medium drought with 13.8% taller than Hunor at 28 DAH at Phase 2 in 2025 under medium drought, 12.7% at 21 DAH Phase 3 in 2024 under moderate drought, whereas Hunor occasionally performed relatively better under mild drought with 3.8% taller at 14 DAH at Phase 3 in 2023. This confirms differential drought-response strategies between genotypes, reflecting genotype-specific plasticity where certain cultivars maintain growth through improved stomatal regulation and biomass allocation under stress (Chen et al., 2016; Edwards et al., 2016). The stronger advantage of Magor under increasing drought intensity suggests greater resilience mechanisms activated under water limitation, confirming its superior adaptive plasticity. Similarly, a significant treatment x season interaction at 21 and 28 DAH across all phases indicates that biostimulant effectiveness was strongly season-dependent. Particularly, T2 consistently produced greater plant height increases under moderate and medium drought compared with mild drought by 12.4 -17.4% at Phase 1 at 21 DAH, 15 -18.7% at Phase 2 at 28 DAH, 12.4 -14.5% at Phase 3 at 21 DAH, and 12.9–21.3% at Phase 4 at 28 DAH over the control. These findings align with previous studies showing that biostimulant efficacy increases under abiotic stress because bioactive compounds enhance osmoprotection, antioxidant enzyme activity, and nutrient assimilation (Bhupenchandra et al., 2022; Ma et al., 2022). The enhanced response during more severe drought seasons supports the concept of stress-triggered biostimulant activation. Overall, these findings contribute to advancing understanding of alfalfa growth dynamics under contrasting drought stress conditions by demonstrating that seasonal climatic variability is the dominant determinant of plant height, followed by inherent genetic differences. Biostimulants provide measurable growth enhancement, particularly during mid- to late-regrowth stages with T2 improvements of 12-28% under moderate/medium drought), but do not fundamentally alter genotype-specific performance patterns across seasons. The results highlight the

importance of integrating genotype selection (favoring Magor under severe drought), seasonal adaptation strategies, and stage-specific management practices to optimize alfalfa productivity under increasing climatic variability.

Table 7: Interactive effect of biostimulant and genotypic variation and crop year on plant height (PH) of alfalfa under contrasting drought stress conditions (Debrecen, 2023-2025)

Phase 1				
Source of Variation	Df	PH @14 DAH	PH@21 DAH	PH@28 DAH
Treatment (B)	3	2.53 ns	107.56**	86.71**
Genotype (G)	1	12.02**	104.34**	116.21**
Season (Y)	2	29.32**	54.47**	257.77**
B x G	3	0.47 ns	0.24 ns	0.12 ns
B x Y	6	1.02 ns	12.54**	1.49 ns
G x Y	2	4.03*	12.96**	12.00**
B x G x Y	6	0.18 ns	0.80 ns	0.87 ns
Error	46			
Phase 2				
Treatment (B)	3	2.50 ns	112.95**	45.92**
Genotype (G)	1	32.48**	109.91**	125.42**
Season (Y)	2	16.11**	196.15**	49.54**
B x G	3	4.03*	1.67 ns	1.57 ns
B x Y	6	0.13 ns	4.88**	0.44 ns
G x Y	2	0.07 ns	5.10**	10.00**
B x G x Y	6	1.03 ns	0.31 ns	1.17 ns
Error	46			
Phase 3				
Treatment (B)	3	0.75 ns	20.58**	53.53**
Genotype (G)	1	7.64*	52.95**	26.94**
Season (Y)	2	4.48*	300.82**	324.62**
B x G	3	0.36 ns	0.62 ns	1.61 ns
B x Y	6	1.16 ns	3.17**	4.48**
G x Y	2	5.25*	6.22**	1.22 ns
B x G x Y	6	0.63 ns	0.13 ns	0.78 ns
Error	46			
Phase 4				
Treatment (B)	3	2.81*	27.55**	37.64**
Genotype (G)	1	2.58 ns	63.61**	52.56**
Season (Y)	2	34.49**	310.08**	149.22**
B x G	3	0.73 ns	0.89 ns	0.45 ns
B x Y	6	0.43 ns	10.17**	3.97**
G x Y	2	0.68 ns	10.41**	14.10**
B x G x Y	6	0.40	2.95**	1.31 ns
Error	46			

ns = not significant ($p > 0.05$) *= $p \leq 0.05$, ** = $p \leq 0.01$, ** 1st phase: from 1st to 2nd cutting; 2nd phase: from 2nd to 3rd cutting; 3rd phase: from 3rd to 4th cutting, 4th phase: from 4th to 5th cutting.

4.1.4 Differential response of NDVI to biostimulant treatments in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023 - 2025)

The normalised difference vegetation index of alfalfa was significantly affected by biostimulant application and genotype, but no interaction effect was detected; however, this significance varied across measuring intervals and the four phases. Biostimulant treatments consistently enhanced NDVI relative to the untreated control across all phases under varying drought stress conditions (**Figure 5**).

During the 2023 cropping season (mild drought stress), Phase 1 showed no significant effect of biostimulant application on NDVI at 14 days after harvest; however, significant differences emerged at 21 and 28 DAH. At these later stages, treatment T2 consistently resulted in the greatest NDVI of 0.75, a 7.1% increase over the control at 21 DAH, while T3 increased NDVI by 6% over the control at 28 DAH, however, the differences among treatments were statistically similar. Phase 2 followed a similar trend, with no significant differences at 14 DAH but distinct treatment effects at 21 and 28 DAH; by 28 DAH, all treatments elevated NDVI by over 7% relative to the control. Biostimulant effects were especially pronounced in Phase 3, where T1, T2, and T3 attained an NDVI of 0.86 at 28 DAH, a 6% increase compared to 0.81 in the control. This pattern extended to Phase 4, with treatments reaching 0.86 NDVI, a 5% increase compared to 0.82 in the control. Collectively, these results indicate that, under mild drought stress, biostimulants promoted enhanced canopy development and higher NDVI throughout the season.

In the 2024 cropping season (moderate drought stress), a consistent temporal pattern was observed across all phases: treatment effects were non-significant at 14 DAH but became highly significant at 21 and 28 DAH. In Phase 1, NDVI values ranged from 0.56 to 0.58, with T1 and T3 showing marginally higher values of 0.58 each compared to 0.57 in the control (T0) at 14 DAH. At 21 DAH, all biostimulant treatments (T1, T2, and T3) achieved an NDVI of 0.76 each an 8.5% increase over 0.70 in T0. At 28 DAH, T1 and T2 recorded the highest values of 0.89 each, followed by T3 with 0.88, reflecting 11% and 10% increases over T0 (0.80), respectively. Phase 2 mirrored Phase 1, with uniform NDVI values across treatments at 14 DAH. Biostimulant-treated plots (T1, T2, and T3) were statistically similar at 21 DAH with NDVI values of 0.73 each, a 4% higher than T0 at 0.70. At 28 DAH, T1, T2, and T3 recorded high values of 0.87, reflecting about 9% improvement over T0 at 0.80. Phase 3 trends were similar: at 21 DAH, T2 produced the highest NDVI of 0.78, followed by T1 and T3 with 0.76 each, compared to T0 (0.71), indicating 11% and 9% increases, respectively. At 28 DAH, all biostimulant treatments

yielded 0.87 NDVI, a 9% increase over T0 at 0.80. In Phase 4, NDVI values were similar across treatments at 14 DAH. At 21 DAH, T3 recorded the highest NDVI of 0.78, followed by T1 and T2 with 0.77 each, with T0 at 0.71. At 28 DAH, T1 and T2 achieved 0.87, followed by T3 with 0.86, reflecting a 6–7% improvement over untreated plots with 0.81.

In 2025 cropping season (medium drought stress), Phase 1, no significant effect was detected at 14 DAH; numerically, T0 had the highest NDVI of 0.53, followed by T1 and T2 with 0.52 each, and T3 with 0.49. At 21 and 28 DAH, treatments significantly affected NDVI: at 21 DAH, T1 and T2 produced the highest values of 0.70 each, a 4.5% increase, followed by T3 with a 3% increase over T0 at 0.67. By 28 DAH, T1, T2, and T3 resulted in similarly high NDVI values of 0.82–0.83, significantly greater than T0 (0.79). Phase 2 at 14 DAH showed non-significant differences; numerically, T2 increased NDVI by 3.2% over T0 (0.61). At 21 and 28 DAH, trends followed Phase 1, with T1, T2, and T3 increasing NDVI by 5.5% over T0; at 28 DAH, T1 and T2 reached 0.87, a 6% increase each, whereas T3 also increased NDVI by 4.8% relative to the control. In Phase 3 at 14 DAH, treatments were non-significant, with NDVI values ranging from 0.59 to 0.60; at 21 and 28 DAH, T1 and T3 increased NDVI by 7%, and T2 by 5.4%, relative to the control. Similarly at 28 DAH, T2 elevated NDVI by 5% over the control. Phase 4 showed significant effects of treatment at 14, 21, and 28 DAH: at 14 DAH T2 and T3 recorded 0.62, T1 0.61, and T0 0.60; T2 outperformed others by 2.7% over the control at 21 DAH. At 28 DAH, T2 and T3 increased NDVI by 3.8% over the control.

Overall, NDVI responses to biostimulant application varied strongly with growth phase across cropping seasons. Biostimulant treatments generally enhanced NDVI at later stages, suggesting that application irrespective of genotype consistently improved vegetation vigor under contrasting drought stress conditions. Across all drought intensities, biostimulants enhanced NDVI compared to the untreated control: 5–7% under mild drought, up to 11% under moderate drought, and 4–7% under medium drought at later growth stages.

Overall, NDVI responses to biostimulant application varied strongly with growth phase across all cropping seasons, with treatments consistently enhancing NDVI relative to the untreated control (T0), particularly at later regrowth stages (21 and 28 DAH) under mild, moderate, and medium drought intensities. For instance, improvements ranged from 5–7% at 28 DAH under mild drought, up to 11% under moderate drought, and 4–7% under medium drought irrespective of genotype. These significant NDVI increases indicate

enhanced canopy development, chlorophyll retention, and photosynthetic performance under drought stress, as biostimulants promote vegetation vigor non-destructively measurable by NDVI.(Carvalho et al., 2015). The consistent delayed treatment response observed at 14 days after harvest (DAH), but differences emerged primarily at 21 and 28 DAH across all the cropping seasons, suggests that biostimulants may enhance metabolic and physiological processes that become more evident during later regrowth stages. This aligns with previous findings that biostimulants improve nutrient uptake efficiency, chlorophyll synthesis, and stress resilience over time rather than immediately after application (Calvo et al., 2024; Akdag et al., 2024; Martinez-Lorente et al., 2024). NDVI improvements were more pronounced under moderate drought stress (2024 season) support the hypothesis that biostimulants are particularly effective under suboptimal but not extreme stress conditions. Under moderate stress, plants may retain sufficient metabolic activity to respond positively to biostimulant-induced hormonal or biochemical stimulation (Bulgari et al., 2019; Rouphael and Colla, 2020). This could explain the observed NDVI increases of up to 11% compared with untreated controls.

Furthermore, the consistent enhancement of NDVI across all phases suggests improved canopy persistence and delayed senescence. An observed increased NDVI at 28 DAH across biostimulant treatments indicates sustained chlorophyll retention and potentially improved water-use efficiency, which are critical traits for drought adaptation (Liatitle, 2021). The frequent superiority of T2 may indicate a more effective formulation or concentration, possibly enhancing osmotic regulation or antioxidant activity under stress conditions.

The lack of a strong treatment x genotype interaction suggests that biostimulant effects were relatively stable across genetic backgrounds. This broad-spectrum effectiveness is advantageous for practical application, as it implies that farmers can expect consistent improvements in canopy vigor regardless of cultivar choice. Overall, these findings demonstrate that biostimulant application significantly enhances alfalfa NDVI, particularly at later regrowth stages, and that its effectiveness persists across varying drought intensities. The results provide strong evidence that biostimulants can serve as a valuable agronomic tool to improve vegetation vigor and drought resilience in alfalfa production systems.

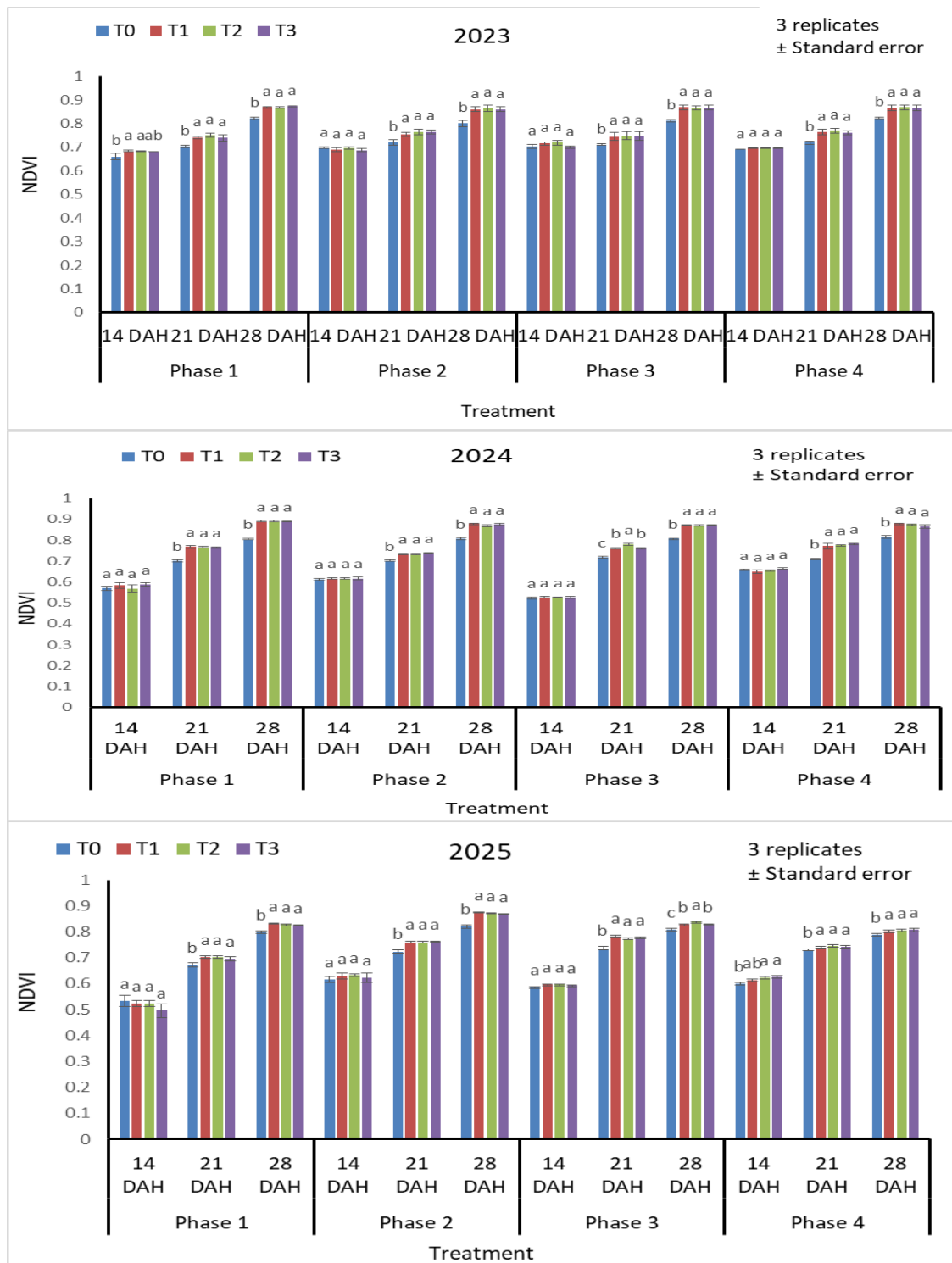


Figure 5: Effect of biostimulant treatments on normalised difference vegetation index (NDVI) of the alfalfa crop during the three cropping seasons (Debrecen, 2023–2025). *Note: Columns represent annual mean values of genotypes (2) and repetitions (3) $\pm$ standard error ($n = 6$). Different letters mean significant differences between treatments at the same phase at $p = 5\%$. ** 1st phase: from 1st to 2nd cutting; 2nd phase: from 2nd to 3rd cutting; 3rd phase: from 3rd to 4th cutting, 4th phase: from 4th to 5th cutting.*

4.1.5 Differential response of NDVI to genotypic variation in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023-2025)

Genotype Magor consistently outperformed Hunor in NDVI across all phases during the 2023 (mild drought stress) cropping season (**Figure 6**). In Phase 1, no significant effect was detected at 14 DAH, as mean values were similar; however, significant effects emerged at both 21 and 28 DAH. At 21 DAH, Magor exhibited a significantly higher NDVI, increasing it by 2.7% over Hunor. From 21 to 28 DAH, Magor reached an NDVI of 0.86, a slight 1% increase compared to 0.85 in Hunor. This difference widened in Phase 2, where Magor sustained higher NDVI values, increasing by 1.2% over Hunor at 14 DAH and by 4.4% and 5.1% at 21 and 28 DAH, respectively. The pattern persisted in Phases 3 and 4, with Magor attaining NDVI values approximately 3.5% and 4.7% higher than Hunor at 28 DAH. These findings demonstrate that Magor exhibits a superior inherent capacity for canopy expansion relative to Hunor under mild drought stress conditions.

In the 2024 (moderate drought stress) cropping season, in Phase 1 at 14 DAH, genotype had a significant effect on NDVI; Magor recorded the highest value of 0.58, a 3.5% higher than 0.56 found in Hunor. No significant effects were detected at 21 or 28 DAH, though Magor numerically increased NDVI by 0 to 0.21% over Hunor. A similar trend occurred in Phase 2: at 14 DAH, genotype significantly affected NDVI, with Magor recording a 1.6% higher than Hunor, but no effects at 21 or 28 DAH. Phase 3 mirrored Phases 1 and 2, with a significant genotypic effect at 14 DAH, where Magor recorded an NDVI value of 0.53, reflecting a 2% higher value than the 0.52 found in Hunor; however, no effects were detected at the later stages. In Phase 4, no significant genotypic effects occurred at 14, 21, or 28 DAH, though Magor numerically outperformed Hunor by 1.3% to 2.1% across these time points.

In the 2025 (medium drought stress) cropping season, in Phase 1, NDVI was significantly influenced by genotype at 14 and 21 DAH but not at 28 DAH. Magor recorded a significantly higher NDVI of 0.55, reflecting a 14% higher than 0.48 found in Hunor at 14 DAH. Magor maintained a 3% advantage at 21 DAH and slightly outperformed Hunor by 0.3% at 28 DAH despite lacking significance. Phase 2 followed similar trends: significant genotypic effects at 14 and 21 DAH but not at 28 DAH, with Magor showing 3.3% higher NDVI over Hunor at 14 DAH and a marginal advantage at 21 DAH. In Phase 3 at 14, 21, and 28 DAH, NDVI was not significantly influenced by genotype, though Magor had slightly higher values from 0.8% to 1% above Hunor. In Phase 4, genotype

significantly influenced NDVI at 21 and 28 DAH. At 14 DAH, no significant difference was detected, but Magor increased NDVI by 2.2% over Hunor; at 21 DAH, Magor showed significantly higher NDVI value of 0.75, reflecting a 2.7% higher than the 0.73 found in Hunor's. At 28 DAH, Magor maintained a higher NDVI of 0.81, reflecting a 3.8% higher over 0.78 found in Hunor. Overall, across all three seasons under contrasting drought stress conditions, NDVI responses to genotypic variation varied strongly across measuring intervals and phases. Our results demonstrate that the superior NDVI performance of Magor across drought intensities suggests a stronger inherent capacity for canopy expansion and maintenance compared to Hunor, aligning with and extending current understanding of alfalfa genotype-dependent canopy responses under water-limited environments (Mouradi et al., 2022). The observed consistent NDVI advantage of Magor across mild, moderate, and medium drought stress conditions supports previous research indicating that NDVI is an effective indicator for differentiating genotypic responses to drought stress (Condorelli et al., 2018; Darwish et al., 2025). Some studies report that NDVI strongly correlates with green biomass, chlorophyll concentration, and canopy vigor under water-limited conditions (Cabrera-Bosquet et al., 2011; Condorelli et al., 2018). The higher NDVI values observed in Magor therefore suggest improved radiation interception, delayed senescence, and potentially greater carbon assimilation capacity compared with Hunor. These findings confirm studies reporting that NDVI is closely associated with green biomass, chlorophyll content, and photosynthetic activity; thus, higher NDVI values indicate improved radiation interception and potential carbon assimilation (Kimaro et al., 2023). We noted that across many phases, especially at 14 DAH, NDVI was significantly affected by genotypic variation, while these differences were often absent at 21 and 28 DAH, implying that genotype effects are growth-stage dependent, particularly under moderate drought stress. Similar growth-stage sensitivity of alfalfa has been reported in drought phenotyping studies, where early-season NDVI measurements are more effective in discriminating genotypes before canopy saturation occurs (Ruud et al., 2019). Under medium drought stress, the magnitude of early-stage NDVI differences increased markedly, with Magor exhibiting up to a 14% higher NDVI over Hunor at 14 DAH in Phase 1. This amplification of alfalfa genotypic differences under stronger stress is consistent with other research indicating that drought severity can enhance phenotypic contrast among genotypes (Quan et al., 2016; Ruud et al., 2019).

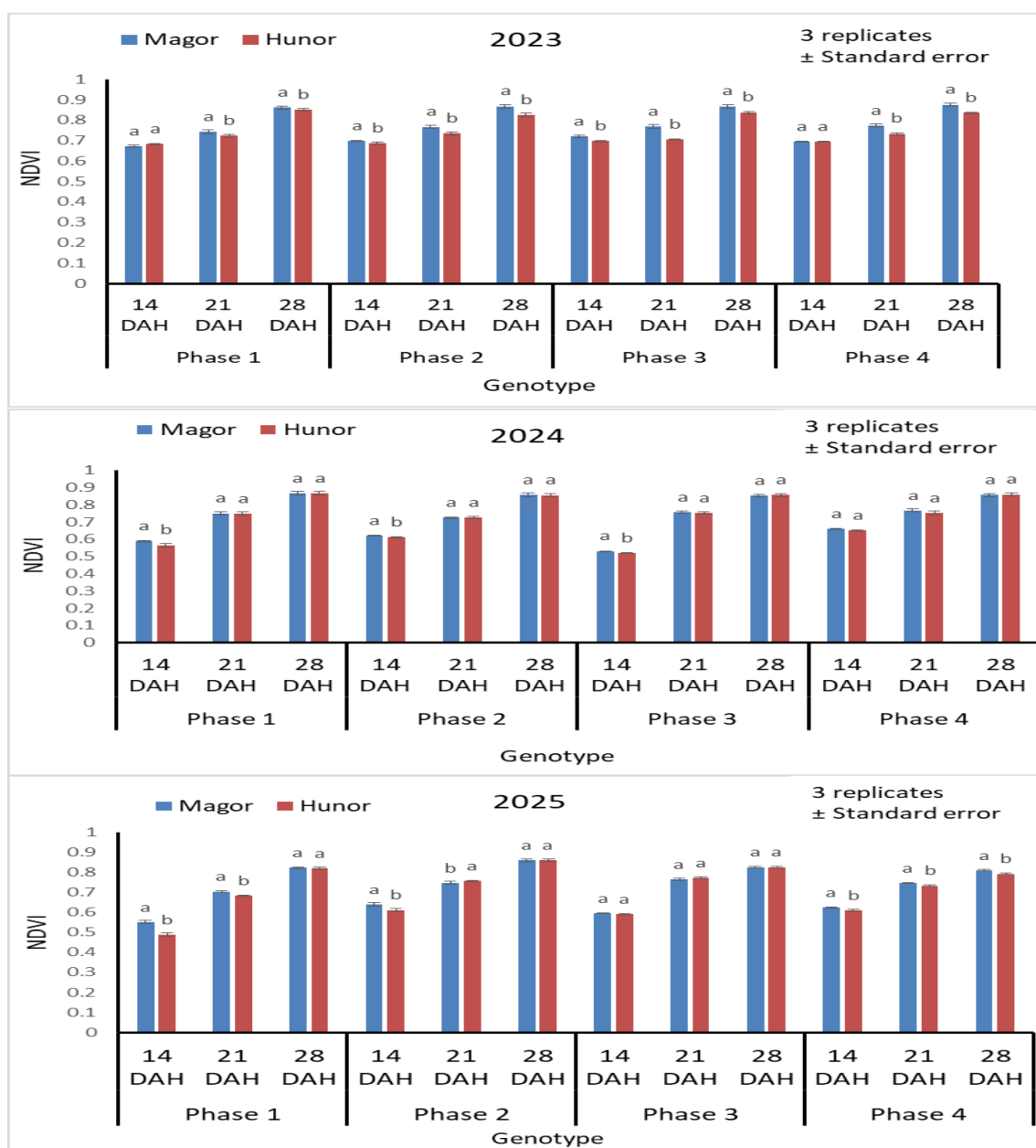


Figure 6: Effect of genotypic variation on normalised difference vegetation index (NDVI) of alfalfa crop during the three cropping seasons (Debrecen, 2023–2025).

*Note: Columns represent annual mean values of treatments (4) and repetitions (3) $\pm$ standard error ($n = 12$). Different letters mean significant differences between genotypes at the same phase at $p = 5\%$. ** 1st phase: from 1st to 2nd cutting; 2nd phase: from 2nd to 3rd cutting; 3rd phase: from 3rd to 4th cutting, 4th phase: from 4th to 5th cutting.*

Other studies have shown that increased stress intensity often highlights canopy reflectance differences, thereby improving discrimination between tolerant and sensitive genotypes (Mohi-Ud-Din et al., 2022). The substantial early advantage of Magor under medium drought suggests superior early vigor and more efficient canopy establishment traits that are critical for reducing soil evaporation and improving water-use efficiency.

In contrast, the decline in NDVI observed in Hunor reflects its lower capacity to maintain canopy integrity under mild, moderate, and medium drought stress conditions. This aligns with studies reporting that drought typically accelerates leaf senescence, reduces chlorophyll content, and limits leaf expansion, all of which reduce NDVI (Moradi et al., 2022). The higher NDVI in Magor suggests better osmotic adjustment and canopy maintenance mechanisms. This trend is consistent with broader studies showing that drought-tolerant genotypes exhibit slower senescence and maintain higher NDVI during stress conditions (Moraga et al., 2023).

4.1.6 Interactive effect of biostimulant and Genotypic variation and crop year on Normalised difference vegetation index (NDVI) of alfalfa under contrasting drought stress conditions (2023-2025)

A comprehensive evaluation of the Biostimulant Treatment x Genotype x Crop Year interaction effect on the Normalized Difference Vegetation Index across all three seasons (2023-2025) under varying drought conditions revealed significant improvements in canopy development and vegetation vigor in alfalfa under abiotic stress (drought) (**Table 8**).

In phase 1 at 14 days after harvest, NDVI was significantly influenced by Genotype and Season, but Treatment had no significant effect. A significant Genotype x Season interaction was observed, indicating genotype performance varied across seasons. Specifically, under mild drought, Hunor increased NDVI by 1.5% over Magor; however, under moderate and medium drought, Magor significantly outperformed Hunor by 4.1% and 13%, respectively. All other interactions, including the three-way interaction, were non-significant.

At 21 DAH, Treatment, Genotype, and Season significantly affected NDVI. A significant Treatment x Season interaction was detected, with treatment responses differing across seasons. Under mild drought, T2 increased NDVI by 7%, followed by T1 and T3 at 5.4% each compared to the control. In the 2024 season, T1 increased NDVI by 10%, followed by T2 and T3 at 9.5% and 9.2%, respectively. A similar trend occurred in 2025, where T1 and T2 increased NDVI by 4.2%, followed by T3 at 3.2% over the control. No significant three-way interaction was observed. At 28 DAH, NDVI was significantly affected by Treatment, Genotype, and Season. A significant Treatment x Season interaction was recorded, while other interactions were non-significant. Under mild drought, T3 increased NDVI by 6%, followed by T1 and T2 at 4.8% each compared to

the control. In 2024, T1 and T2 increased NDVI by 11%, followed by T3 at 10% over the control. In 2025, T1 increased NDVI by 5%, followed by T2 and T3 at 3.7% compared to the control.

In the phase 2 at 14 DAH, Genotype and Season significantly influenced NDVI, whereas Treatment had no significant effect. A significant Treatment x Genotype interaction indicated genotype-specific treatment responses. Specifically, T3 increased NDVI by 1.5% in Magor, while T1 and T2 improved NDVI by 1.5% each in Hunor over the control. At 21 DAH, Treatment, Genotype, and Season significantly affected NDVI. A significant Genotype x Season interaction suggested differential genotype performance across seasons. Under mild drought, Magor increased NDVI by 4.1% over Hunor; under moderate drought, both genotypes were similar; and under medium drought, Hunor increased NDVI by 1.3% over Magor. At 28 DAH, all main effects were highly significant. A significant Genotype x Season interaction was detected, while the three-way interaction was non-significant. Magor recorded a 5% increase in NDVI over Hunor under mild drought, whereas both genotypes performed similarly under moderate and medium drought.

In the phase 3 at 14 DAH, Treatment, Genotype, and Season significantly influenced NDVI. A significant Genotype x Season interaction was observed, while other interactions were non-significant. Magor significantly increased NDVI by 4.3% under mild drought, 1.9% under moderate, and 1.7% under medium drought over Hunor. At 21 DAH, all main effects were highly significant, with notable two-way interactions and a significant three-way interaction, indicating treatment effects depended on genotype and season. In 2023, all treatments significantly increased NDVI in Magor by 9.8%, while T2 increased it by 1.4% in Hunor over the control. In 2024, T2 stood out, increasing NDVI by 8% in Magor and 8.4% in Hunor. In 2025, T1 increased NDVI by 6.8% in Magor, while T1 and T3 increased it by 5.1% in Hunor. At 28 DAH, all main effects were significant, along with Treatment x Season, Genotype x Season, and three-way interactions, suggesting complex combined effects. In 2023, all treatments significantly increased NDVI in Magor by 8.6%, and T1 increased it by 4.9% in Hunor. In 2024, T2 increased NDVI by 7.4% in Magor, while T1 and T3 increased it by 8.7% in Hunor. In 2025, T1 and T3 slightly increased NDVI by 2.5% in Magor, while T1 and T2 improved it by 3.7% in Hunor.

In the phase 4, at 14 DAH; all main effects were highly significant, with Treatment x Season and Genotype x Season interactions, but no three-way interaction. For Treatment

x Season, treatments were similar in 2023; T3 increased NDVI by 1.5% in 2024, and T2 and T3 elevated it by 3.3% over the control. Genotypes performed similarly in 2023, while Magor showed 1.5% and 3.3% increases over Hunor in 2024 and 2025, respectively.

At 21 DAH, all main effects were significant, with Treatment x Season and Genotype x Season interactions but no three-way interaction. For Treatment x Season, T2 slightly outperformed others by increasing NDVI by 8.4% over T0 in 2023; T3 increased it by 9.8% in 2024, and T1–T3 elevated it by 1.3% over the control. Magor outperformed Hunor by 5.4% in 2023 and 1.3% each in 2024 and 2025. At 28 DAH, all main effects were significant, with Treatment x Genotype, Treatment x Season, and Genotype x Season interactions but no three-way interaction. For Treatment x Season, all treatments increased NDVI by 4.8% over T0 in 2023; T1 and T2 increased it by 7.4% in 2024, and T1–T3 elevated it by 2.5% over the control. Magor outperformed Hunor by 5% in 2023 and 1.3% in 2025, while Hunor slightly outperformed Magor by 1.2% in 2024.

Overall, our results demonstrate that NDVI was strongly influenced by Season across all phases at three measuring intervals (14, 21 and 28 DAH), indicating that environmental conditions played a major role in canopy development and vegetation vigor. Notably, the significant three-way interaction observed at phase 3 at both 21 and 28 DAH indicates that T1, T2 and T3 effects on NDVI were not uniform but depended on both genotype and seasonal conditions. In 2023, all treatments significantly increased NDVI in Magor by 9.8% at 21 DAH and 8.6% at 28 DAH, while T2 boosted Hunor by 1.4% at 21 DAH; in 2024, T2 increase both genotypes by 8% in Magor and 8.4% in Hunor at 21 DAH; whereas T1 and T3 led improvements in Hunor in 2025. This underscores how biostimulant-induced physiological responses in alfalfa canopy such as enhanced photosynthetic activity and vigor are modulated by genotype traits and environmental variability, with greater efficacy under drought stress. These results align with prior research showing biostimulants improve canopy growth and biomass most effectively under abiotic stresses like drought, outperforming non-stressed conditions (Akdağ et al., 2024). Furthermore, the variability in NDVI measurements across growth stages and genotypic responses highlights its utility as a diagnostic tool for assessing plant vigor and treatment efficacy under varying environmental pressures (Cabrera-Bosquet et al., 2011). The consistent performance of T1, T2, and T3 in improving NDVI over the control across nearly all genotype–season combinations increases from 4.8–11% in Phase 1; 1.5 – 4.3% in the Phase 2 and 4, and up to 9.8% in Phase 3 confirms their positive effects of

biostimulant treatments on alfalfa canopy vigor (Akdağ et al., 2024). These treatments enhanced vegetation greenness and canopy density, particularly under mild to medium drought stress. The observed NDVI improvements can be attributed to enhanced photosynthetic efficiency and biomass accumulation (Melash and Ábrahám, 2022), consistent with findings that biostimulants increase chlorophyll content, thereby boosting light absorption and photosynthetic capacity in alfalfa (Appiah et al., 2024a). Such enhancements are critical for maintaining plant health and productivity, aligning with significant genotypic effects on multispectral traits like NDVI observed in drought-stressed crops (Burner et al., 2025). The varying responses across genotypes and seasons underscore the need for genotype-specific biostimulant formulations to optimize agronomic outcomes and drought tolerance in alfalfa. Seasonal influences were pronounced, with NDVI declining across all treatments and genotypes in the medium drought-stressed 2025 season, limiting overall treatment efficacy compared to milder conditions in 2023–2024. Similar patterns have been reported, where adverse environmental conditions diminish biostimulant efficacy on photosynthetic parameters (Filho et al., 2024). This emphasizes environmental monitoring and adaptive management strategies to maximize biostimulant benefits amid climatic variability (Larsen et al., 2024). Inter-varietal differences further reinforce genotype-specific stress adaptation mechanisms (Zhang et al., 2025), as seen in Magor's superior NDVI under mild to medium drought. Magor generally exhibited higher NDVI than Hunor under mild–moderate drought by 5% at Phase 2, 4.3% at Phase 3 at 14 DAH, reflecting better biostimulant responsiveness. However, performance converged in the medium drought stress in 2025 season, indicating environmental stress constrained genotypic expression and imposed a maximum on biostimulant efficacy when physiological limits are exacerbated. This aligns with evidence that biostimulants variably maintain chlorophyll under severe stress across cultivars (Kałużewicz et al., 2017; Sosnowski et al., 2019; Silva et al., 2023). Overall, NDVI responses proved growth-stage dependent, peaking in complexity during Phase 3 a critical period where genotype–treatment interactions are amplified by seasonal conditions.

Table 8: interactive effect of biostimulant and genotypic variation and crop year on normalised difference vegetation index (NDVI) of alfalfa under contrasting drought stress conditions (Debrecen, 2023-2025)

Source of Variation	Df	Phase 1		
		NDVI @14 DAH	NDVI @21 DAH	NDVI @28 DAH
Treatment (B)	3	0.53 ns	37.15**	199.43**
Genotype (G)	1	20.85**	14.1**	4.18*
Season (Y)	2	269.95**	83.64**	231.26**
B x G	3	0.34 ns	0.22 ns	1.17 ns
B x Y	6	2.24 ns	2.73*	18.61**
G x Y	2	14.35**	2.75 ns	2.07 ns
B x G x Y	6	1.32 ns	0.08 ns	0.42 ns
Error	46			
Source of Variation	Df	Phase 2		
		NDVI @14 DAH	NDVI @21 DAH	NDVI @28 DAH
Treatment (B)	3	0.45 ns	38.12**	89.65**
Genotype (G)	1	17.24**	6.0*	22.09**
Season (Y)	2	148.35**	27.68**	6.2**
B x G	3	3.37*	0.1 ns	0.6 ns
B x Y	6	0.48 ns	0.39 ns	0.85 ns
G x Y	2	2.29 ns	17.28**	18.7**
B x G x Y	6	0.85 ns	0.81ns	0.12 ns
Error	46			
Source of Variation	Df	Phase 3		
		NDVI @14 DAH	NDVI @21 DAH	NDVI @28 DAH
Treatment (B)	3	3.09*	83.68**	129.97**
Genotype (G)	1	20.9**	70.09**	17.27**
Season (Y)	2	1530.48**	56.13**	87.13**
B x G	3	0.37 ns	7.38**	0.78 ns
B x Y	6	0.93 ns	3.18**	10.3**
G x Y	2	3.62*	84.16**	24.81**
B x G x Y	6	0.31 ns	4.98**	3.37*
Error	46			
Source of Variation	Df	Phase 4		
		NDVI @14 DAH	NDVI @21 DAH	NDVI @28 DAH
Treatment (B)	3	7.14**	45.2**	58.78**
Genotype (G)	1	10.72**	54.81**	48.79**
Season (Y)	2	424.76**	15.04**	219.13**
B x G	3	2.3 ns	1.03 ns	3.14*
B x Y	6	2.72*	6.77**	6.53**
G x Y	2	3.63*	7.47**	23.24**
B x G x Y	6	0.99 ns	0.33 ns	0.89 ns
Error	46			

Note: ns = not significant ($p > 0.05$) * = $p \leq 0.05$, ** = $p \leq 0.01$, ** 1st phase: from 1st to 2nd cutting; 2nd phase: from 2nd to 3rd cutting; 3rd phase: from 3rd to 4th cutting, 4th phase: from 4th to 5th cutting.

4.1.7 Effect of biostimulants treatments and genotype variability on net photosynthetic rate of *Medicago sativa* L. and *Medicago x varia* Martyn

Analysis across all four experimental phases demonstrated that biostimulant applications, alfalfa genotypes, and their interactions exerted highly significant influences on net photosynthetic rate at 14 and 28 days after harvest. Although the intensity of these effects varied across phases, distinct treatment-by-genotype patterns were consistently apparent throughout the experiment (**Table 9**).

In the 2023 (mild drought stress) cropping season, significant interactive effects were detected across phases at different measuring intervals. In Phase 1, the highest net photosynthetic rates of $23.38 \mu\text{mol m}^{-2} \text{s}^{-1}$ were observed in Magor under T0, followed by T1 with $23.50 \mu\text{mol m}^{-2} \text{s}^{-1}$ in Hunor at 14 DAH; at 28 DAH, Magor yielded the maximum rate of $22.68 \mu\text{mol m}^{-2} \text{s}^{-1}$ under T2, reflecting a 71% increase relative to the control. In Phase 2, the maximum net photosynthetic rates of $34.88 \mu\text{mol m}^{-2} \text{s}^{-1}$ was observed in Hunor under T0, followed by T1 in Hunor with $29.62 \mu\text{mol m}^{-2} \text{s}^{-1}$ and T2 in Magor with $25.23 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 14 DAH. At 28 DAH, T1 increased net photosynthetic rates by 10% in Hunor, followed by a 68% increase in Magor under T2. These findings suggest that T2 promoted photosynthesis under mild drought stress. In Phase 3, T2 in Magor recorded the highest net photosynthetic rates of $25.96 \mu\text{mol m}^{-2} \text{s}^{-1}$, while Hunor under T0 achieved $25.16 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 14 DAH. At 28 DAH, T3 enhanced the net photosynthetic rates by 8% in Magor and 5% in Hunor under T2 relative to the control. Overall, T2 and T3 consistently elicited the most favorable physiological outcomes, notably in Magor under mild drought stress. In Phase 4, the maximum net photosynthetic rates were observed under T0 in Magor, followed by T2 with $2.34 \mu\text{mol m}^{-2} \text{s}^{-1}$ in Hunor at 14 DAH. At 28 DAH, T2 recorded the maximum value of $33.7 \mu\text{mol m}^{-2} \text{s}^{-1}$, followed by T3 in Hunor with $29.69 \mu\text{mol m}^{-2} \text{s}^{-1}$, reflecting 24% and 10% increases relative to the control, respectively. These results further underscore the sustained benefits of T2, particularly for Hunor.

In the 2024 (moderate stress drought) cropping season, significant interactive effects were also detected across phases at different measuring intervals. In Phase 1, the maximum net photosynthetic rate of $24.69 \mu\text{mol m}^{-2} \text{s}^{-1}$ was observed in Hunor under T0, followed by Magor under T0 with $23.51 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 14 DAH. At 28 DAH, T2 increased net photosynthetic rates by 14.5% in Hunor, followed by a slight 2% increase in Magor under T3 relative to the control. Collectively, T2 proved most advantageous for Hunor, enhancing physiological performance under moderate drought stress. In Phase 2, the

maximum net photosynthetic rates of $20.86 \mu\text{mol m}^{-2} \text{s}^{-1}$ was observed in Magor under T1 at 14 DAH, reflecting a 9% increase relative to the control, whereas T0 outperformed other treatments in Hunor, suggesting a negative effect of biostimulants. At 28 DAH, T3 increased the net photosynthetic rates by 54% in Hunor, followed by 11% in Magor under T1 relative to the control. In Phase 3, T1 significantly increased the net photosynthetic rates by 86% in Magor, followed by 6% in Hunor under T2 relative to the control at 14 DAH. At 28 DAH, T2 and T1 increased net photosynthetic rates by 7% and 5% in Magor, respectively, with a slight 1.2% increase in Hunor under T2. In Phase 4, T2 significantly increased net photosynthetic rates by 76% in Magor and 2% in Hunor at 14 DAH relative to the control. At 28 DAH, T3 and T2 increased net photosynthetic rates by 27% and 22% in Magor relative to the control, whereas T0 outperformed other treatments in Hunor.

In the 2025 (medium drought stress) cropping season, significant interactive effects were detected across phases at different measuring intervals, except at 14 DAH in Phase 3. In Phase 1, T0 under both Magor and Hunor outperformed all other treatments at 14 DAH, suggesting a negative effect on net photosynthetic rate during regrowth under medium drought stress. However, at 28 DAH, T1 increased the net photosynthetic rates by 11.8% in Magor, followed by 14% in Hunor under T2 relative to the control. In Phase 2, T1 increased the net photosynthetic rates by 8.4% in Magor, followed by 8.8% in Hunor under T2 at 14 DAH relative to the control. Both treatments continued strong performance at 28 DAH, with T1 improving the net photosynthetic rates by 21.9% in Magor and T2 by 4.6% in Hunor relative to the control. In Phase 3, although no significant effects were detected, T2 numerically enhanced the net photosynthetic rates by 1.4% in Hunor, whereas a slight decline was observed in Magor at 14 DAH relative to the control. At 28 DAH, T3 enhanced the net photosynthetic rates by 15% in Hunor, followed by 5% under T2, with a slight 0.9% increase in Magor under T2 relative to the control. In Phase 4, T1 significantly increased the net photosynthetic rates by 21% in Magor, followed by a slight 3.4% increase in Hunor under T3 relative to the control. A similar trend was noted at 28 DAH, where T1 enhanced the net photosynthetic rates by 6% in Hunor, followed by 6% and 4% increases in Magor under T1 and T3, respectively, relative to the control.

Across all three cropping seasons (2023-2025), our study demonstrated highly consistent significant interactive effects between biostimulant treatments and alfalfa genotypes on net photosynthetic rate under mild, moderate, and medium drought stress. The magnitude and direction of treatment responses were strongly influenced by drought intensity, genotype, and regrowth stage. However, our findings show that biostimulant treatments

(T1, T2, and T3) enhance the photosynthetic performance of the two alfalfa genotypes under drought stress by improving stomatal conductance, chlorophyll content, and carbon assimilation efficiency. These findings align with studies that reported that biostimulants improved photosynthetic activity and stress resilience across several crops, particularly under moderate water limitation (Calvo et al., 2014; Rouphael and Colla, 2021). Similarly, some studies demonstrated that biostimulants enhance physiological parameters, including net photosynthetic rate and water use efficiency, through hormonal modulation and improved antioxidant capacity (Bulgari et al., 2019; Rouphael and Colla, 2021; Bhupenchandra et al., 2022; El Moukhtari et al., 2024). The consistent delays at 14 DAH and sustained improvements observed at 28 DAH under stress conditions across the study align with reports that biostimulants often stimulate recovery mechanisms rather than providing immediate stress alleviation (Rouphael and Colla, 2021; Ma et al., 2022; Akdag et al., 2024). Comparatively, the stronger effects of the treatments at 28 DAH compared to 14 DAH under treatment application further support the notion that biostimulants enhance metabolic adjustment and recovery processes over time. Furthermore, the substantial increase of 71% by T2 in Magor under mild drought and the 86% and 21% increase in net photosynthetic rate observed in Magor under T1 during both moderate and medium drought are consistent with findings by studies that reported that protein hydrolysate-based biostimulants can markedly enhance carbon assimilation under stress by stimulating nitrogen metabolism and enzyme activity (Van Oosten et al., 2017; Baltazar et al., 2021; Malecange et al., 2023). Similarly, Wang et al. (2022) demonstrated the regulation of stomatal aperture under protein hydrolysates, allowing for sustained carbon assimilation under mild stress. In contrast, while many studies report uniform improvements across cultivars or genotypes, our results highlight strong genotype-dependent responses: specifically, Magor responded more consistently to T1 and T2 under contrasting drought stress conditions, whereas Hunor exhibited more selective responses. This corroborates studies suggesting relatively genotype-independent effects of biostimulants (González-Morales et al., 2021). Rouphael and Colla, (2021) emphasized that plant genetic background strongly influences responsiveness due to differences in hormonal sensitivity and stress signaling pathways. Therefore, our findings reinforce this concept, showing that the magnitude of photosynthetic enhancement varied substantially between Magor and Hunor across these contrasting drought stress conditions. In addition, we occasionally observed superior performance of the untreated control, particularly at 14 DAH under medium drought stress, indicating early negative or neutral responses to

treatment and demonstrating a potential transient metabolic cost associated with biostimulant application under higher stress intensity. This finding aligns with more recent perspectives suggesting that biostimulant efficacy may depend on stress severity thresholds, beyond which metabolic stimulation may initially burden already stressed plants (Akdag et al., 2024).

Table 9: Effect of biostimulant and genotypic variation on net photosynthesis rate of alfalfa under contrasting drought conditions (Debrecen, 2023-2025)

2023	Phase 1				Phase 2				Phase 3				Phase 4			
	14 DAH		28 DAH		14 DAH		28 DAH		14 DAH		28 DAH		14 DAH		28 DAH	
	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor
T0	23.38	23.44	13.22	25.93	22.15	34.88	15.29	25.36	6.99	25.16	25.95	23.75	8.03	2.06	2.69	27.09
T1	21.38	23.50	12.99	23.28	25.07	29.62	19.40	27.92	16.67	24.09	24.85	23.76	4.48	1.68	19.43	26.92
T2	19.65	15.18	22.68	22.41	25.23	25.23	25.66	23.75	25.96	17.59	25.36	24.85	4.05	2.34	21.69	33.71
T3	23.27	17.04	17.51	19.70	24.06	21.00	24.85	23.76	17.18	21.99	27.92	20.12	3.58	1.86	23.93	29.69
Lsd (5%)	2.05		3.63		4.78		3.69		5.28		5.01		0.47		4.67	
2024	14 DAH		28 DAH		14 DAH		28 DAH		14 DAH		28 DAH		14 DAH		28 DAH	
	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor
	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor
T0	23.51	24.69	27.04	20.85	19.06	19.57	5.56	2.35	18.04	24.24	37.29	38.55	22.56	38.35	28.04	32.42
T1	19.90	16.77	20.96	19.39	20.86	13.74	6.16	2.03	33.66	24.62	39.34	35.88	15.28	35.88	16.26	31.41
T2	22.18	13.53	22.80	23.88	19.91	19.00	3.71	2.90	28.77	25.66	39.76	39.05	39.76	39.05	34.26	26.16
T3	22.74	13.91	27.66	20.82	18.81	16.45	3.79	3.62	28.55	17.91	29.76	35.41	34.88	35.41	35.54	21.20
Lsd (5%)	2.71		3.47		3.21		1.46		5.87		4.04		4.51		3.87	
2025	14 DAH		28 DAH		14 DAH		28 DAH		14 DAH		28 DAH		14 DAH		28 DAH	
	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor
	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor	Magor	Hunor
T0	45.47	43.88	35.65	35.46	24.73	25.35	17.48	21.26	33.57	28.85	26.36	21.19	26.44	29.94	28.08	28.21
T1	50.85	42.50	28.40	34.46	26.81	19.70	21.31	18.33	32.36	25.43	25.24	21.53	32.12	29.55	28.48	29.89
T2	41.91	50.03	30.12	32.45	22.99	27.60	17.78	31.15	33.47	29.27	26.62	22.21	29.43	29.83	29.12	27.18
T3	49.68	42.93	30.87	32.18	24.69	27.11	15.00	35.10	31.27	28.84	24.50	24.42	23.09	30.98	29.74	20.75
Lsd (5%)	4.30		2.79		2.90		2.48		3.08		4.03		3.02		3.84	

Note: DAH = Days after harvest, ** 1st phase: from 1st to 2nd cutting; 2nd phase: from 2nd to 3rd cutting; 3rd phase: from 3rd to 4th cutting, 4th phase: from 4th to 5th cutting.

4.1.8 Three-way interactive effect of biostimulant treatment x genotypic variation x crop year (environment) on net photosynthetic rate of alfalfa under drought stress conditions (2023-2025)

A comprehensive evaluation revealed a highly significant ($p < 0.01$) Treatment x Genotype variation x crop year interaction (Environment) effect on net photosynthetic rate. This interaction exhibited large effect sizes, with partial values ranging from 0.03 to 0.30 for measurements taken at 14 and 28 days after harvest (**Table 10**). These findings indicate that genotype-specific responses to treatments were strongly influenced by year-to-year environmental variation. In other words, the magnitude and direction of treatment effects varied substantially across crop years, reflecting environmental modulation of physiological performance in alfalfa crop cultivation. This highlights the predominant role of year-dependent conditions in alfalfa photosynthetic efficiency and suggests limited temporal stability of treatment responses across growing seasons. Such variability in biostimulant efficacy aligns with observations in other species, where temperature and

rainfall can mitigate or exacerbate their effects (Franzoni et al., 2023). Specifically, the statistically significant divergence in photosynthetic traits across different crop years and genotypes further substantiates the complex genotype x environment (G x E) interplay (Zhang et al., 2024). This suggests that while individual variables such as biostimulant application significantly influence net photosynthetic rate, their ultimate impact is intricately linked to the genetic background of the alfalfa variety and prevailing environmental conditions of a given crop year (Antonucci et al., 2021; Zheng et al., 2023). Environmental factors modulate biostimulant efficacy by influencing physiological processes, nutrient uptake, and plant health under stress (Franzoni et al., 2023). Moreover, significant correlations between physiological parameters such as plant height, SPAD readings (chlorophyll content), and fresh herbage yield underscore complex interdependencies in responses to biostimulants and environmental conditions (Appiah et al., 2024a). Collectively, these results demonstrate that alfalfa photosynthetic efficiency is highly plastic, responding dynamically to environmental fluctuations. This plasticity confers adaptive advantages but also introduces variability in agronomic performance under contrasting drought stress conditions. Overall, these findings emphasize that environmental variability is the dominant determinant of biostimulant treatment responses, underscoring the need to incorporate such environmental variability into research frameworks that contribute to more resilient alfalfa production systems, improved input-use efficiency, and enhanced economic sustainability for farmers operating under increasingly variable climatic conditions.

Table 10: Biostimulant treatment, genotype variation and crop year interaction effect on photosynthetic rate of *Medicago sativa* L. and *Medicago x varia* Martyn under drought stress condition (Debrecen, 2023-2025)

Parameter	D.F	F	p	Partial η^2
14 NPR at phase 1	6	12.10	< 0.001	0.15
28 NPR at phase 1	6	6.35	< 0.001	0.08
14 NPR at phase 2	6	6.92	< 0.001	0.09
28 NPR at phase 2	6	30.40	< 0.001	0.30
14 NPR at phase 3	6	7.99	< 0.001	0.10
28 NPR at phase 3	6	2.81	0.011	0.03
14 NPR at phase 4	6	5.00	< 0.001	0.06
28 NPR at phase 4	6	6.37	< 0.001	0.08

Note: 14, 28 = days after harvest, NPR = net photosynthetic rate, d.f = degree of freedom, F = F-value. P = significance, partial η^2 = partial eta square

4.2 Effect of biostimulants treatments, genotype variability and environment on dry matter yield of *Medicago sativa* L. and *Medicago x varia* Martyn

4.2.1 Differential effects of biostimulant treatments on dry matter yield in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023-2025)

Before we conducted the statistical analysis, all the harvested yield data were standardised to the recommended moisture content of 15%. This standardisation was necessary because moisture content varied among plots at the time of harvesting and measurement. Therefore, adjusting yields to a common moisture basis ensured a valid comparison of dry matter yield production across treatments, genotypes, and environments (crop year). The effects of biostimulant treatments on dry matter yield of alfalfa under different drought intensities are presented in **Figure 7**. Under mild drought (2023), the statistical analysis revealed that phase 1, phase 2 and phase 3 showed no significant differences ($p > 0.05$) among treatments, however, phase 4 exhibited significant differences ($p \leq 0.05$), where T2 produced the highest dry matter yield of 8956 kg ha⁻¹ followed by T1 and T3 with yield of 8815 kg ha⁻¹ and 7672 kg ha⁻¹ whereas T0 recorded the least yield. Under moderate drought (2024) the biostimulant treatment improved dry matter yield at phase 1 to phase 4 respectively when compared to the control. In addition, T2 numerically outperformed all the remaining treatment levels (T1 and T3) although they were statistically similar. Similarly, under medium drought (2025) the biostimulant treatment enhance dry matter yield at phase 1 to phase 4 when compared to the control. Comparatively, T2 numerically outperformed all the remaining treatment levels (T1 and T3) although they were statistically similar.

Across the three seasons, dry matter yield values ranged from 5851 kg ha⁻¹ under the control treatment (T0) in 2025 to 7414 kg ha⁻¹ under T2 in 2024. The relatively uniform response suggests that early regrowth after cutting was less influenced by biostimulant application and more dependent on baseline soil fertility and climatic conditions. However, across growing seasons, T2 was found to increase dry matter by 6% in 2023 and 17% in 2024, whereas T3 in 2025 increased dry matter yield by 22% compared to its respective control groups.

The highest dry matter yield of 8527 kg ha⁻¹ was observed in 2024 under T2, followed closely by T1 in the same year with 8041 kg ha⁻¹. These higher yields may be attributed to the enhanced physiological activity promoted by biostimulants under favorable seasonal conditions in 2024, such as improved nutrient uptake and photosynthetic efficiency. The lowest dry matter yield of 4932 kg ha⁻¹ was observed in 2023 under T1,

indicating that biostimulant effectiveness may be limited under less favorable environmental conditions or during periods of stress.

Conversely, despite noticeable numerical differences, phase 3 did not show statistically significant variation among treatments. Nevertheless, the highest dry matter yield of 10,283 kg ha⁻¹ was observed in 2025 under T2, while the minimum dry matter yield of 5225 kg ha⁻¹ was also observed under T0 in 2025. The lack of statistical significance suggests that variability among replications or environmental factors may have masked treatment effects, even though T2 consistently produced higher numerical yields, likely due to cumulative biostimulant effects over successive harvests or phases.

Lastly, significant differences were again evident at phase 4 under mild drought indicates that the effect of applied treatments became more evident when water availability is limited. The highest dry matter yield of 8956 kg ha⁻¹ was observed in 2023 under T2, closely followed by T1 in the same year with a dry matter yield of 8815 kg ha⁻¹. This response may be related to the role of biostimulants in sustaining plant vigour and delaying senescence during later harvests. Conversely, the lowest dry matter yield of 3740 kg ha⁻¹ was observed in 2025 under T0, likely due to nutrient depletion and cumulative cutting stress in the absence of biostimulant support. Overall, across significant differences observed at phases 2 and 4, T2 consistently produced the highest dry matter yield, particularly in 2024 and 2023, highlighting its superior effectiveness in enhancing biomass production under favourable seasonal conditions. The control treatment (T0) generally produced the lowest yields, especially in later phases, emphasising the importance of biostimulant application in sustaining productivity over multiple harvests.

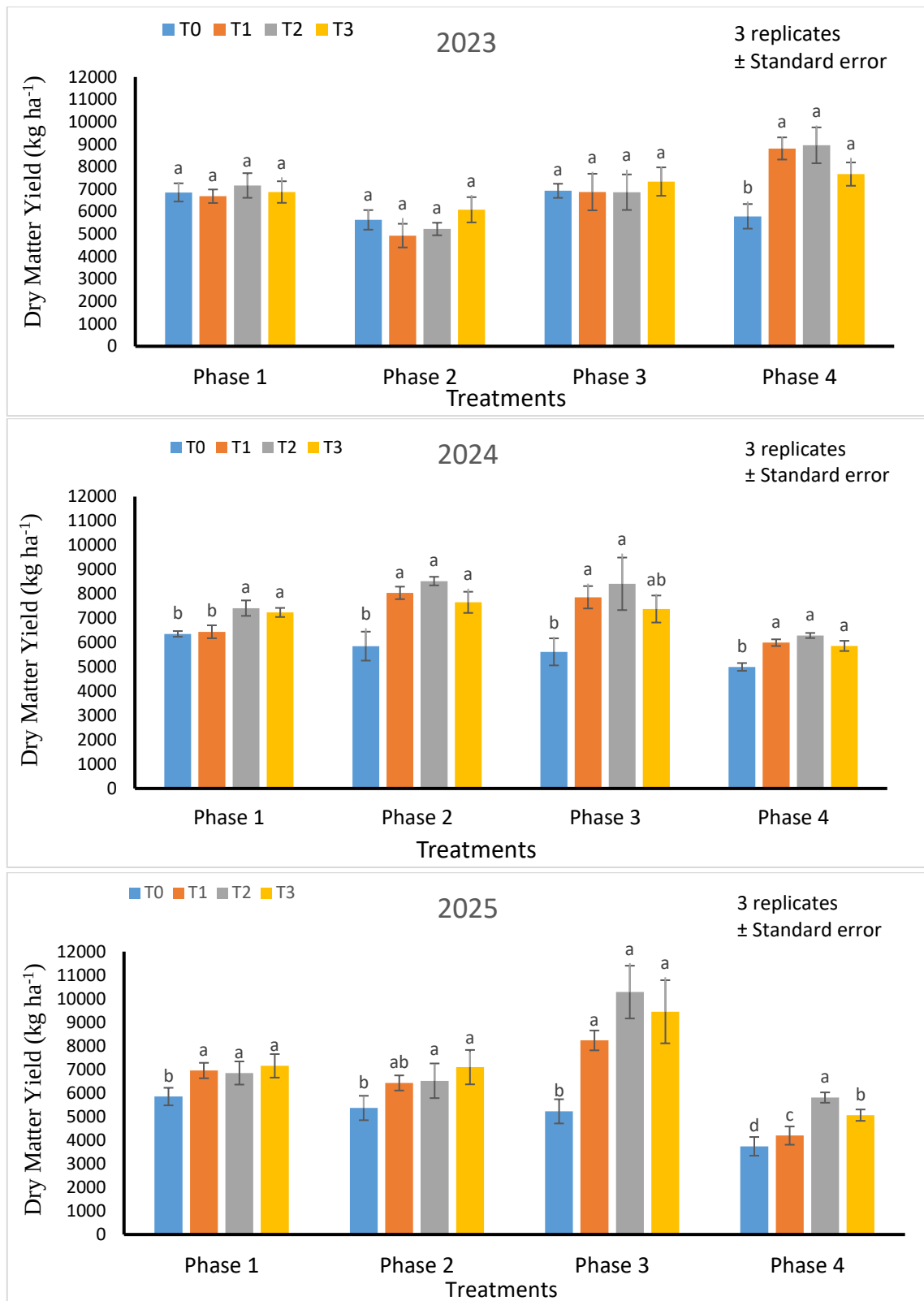


Figure 7: Effect of biostimulant treatments on dry matter yield (kg ha^{-1}) of alfalfa crop during the three cropping seasons (Debrecen, 2023–2025).

Note: Columns represent annual mean values of genotypes (2) and repetitions (3) ± standard error ($n = 6$). Different letters mean significant differences between treatments at the same phase at $p = 5\%$. ** 1st phase: from 1st to 2nd cutting; 2nd phase: from 2nd to 3rd cutting; 3rd phase: from 3rd to 4th cutting, 4th phase: from 4th to 5th cutting.

4.2.2 Differential effects of genotypic variation on dry matter yield in *Medicago sativa* L. and *Medicago x varia Martyn* across three cropping seasons (2023-2025)

The effects of genotype variation on dry matter yield of alfalfa across four phases under different drought intensities are presented in **Figure 8**. Under mild drought (2023), the effectiveness of the genotypes was not consistent; statistically, no effect of the genotype existed at phase 1 and phase 3; however, a significant influence was observed at phase 2 and phase 4, respectively. Under moderate drought (2024), the genotypic variation improved dry matter yield at phases 1 to 3, except phase 4. Similarly, under medium drought (2025), the genotypic variation enhanced dry matter yield from phase 1 to phase 4. Comparatively, dry matter yield in phase 1 varied significantly among genotypes across the individual drought intensities. The highest dry matter yield of 7439 kg ha⁻¹ was observed in *Medicago x Varia Martyn* (Magor) during 2025, while the lowest dry matter yield of 5967 kg ha⁻¹ was also observed in *Medicago Sativa* L (Hunor) during 2025. Across all the different drought intensities, variegated alfalfa consistently outperformed Hunor. The superior performance of variegated alfalfa may be attributed to its better early-season vigour and higher adaptability to seasonal conditions, whereas the lower dry matter yield of Hunor suggests comparatively reduced growth potential during the first phase.

In phase 2, significant differences were also evident across the individual drought intensities, where the highest dry matter yield of 7678 kg ha⁻¹ was observed in variegated alfalfa in 2024, while the lowest dry matter yield of 4939 kg ha⁻¹ was observed in Hunor in 2023. The marked increase in yield for variegated alfalfa in 2024 could be linked to favourable climatic conditions during regrowth, such as adequate moisture and optimal temperatures. Conversely, the low dry matter yield of Hunor in 2023 may be due to poor regrowth ability and higher sensitivity to seasonal stress.

Although numerical differences were observed among genotypes in phase 3 across the individual drought intensities, these differences were not statistically significant. Nonetheless, the highest numerical dry matter yield of 9043 kg ha⁻¹ was observed in variegated alfalfa in 2025, whereas the lowest dry matter yield of 6300 kg ha⁻¹ was also observed under Hunor in 2024. The lack of significance suggests that environmental factors during this growth phase may have minimised genotypic differences, leading to more uniform performance across treatments.

Finally, across the individual drought intensities at phase 4, the highest dry matter yield of 8673 kg ha⁻¹ was achieved by variegated alfalfa in 2023, whereas the lowest dry matter yield of 4113 kg ha⁻¹ was also observed in Hunor in 2025. The superior performance of variegated alfalfa in 2023 may be associated with its sustained persistence and better tolerance to late-season conditions. In contrast, the sharp decline in Hunor yield in 2025 likely reflects cumulative stress effects, such as nutrient depletion and reduced regrowth capacity at the final phase. Overall, the results demonstrate that the dry matter yield of alfalfa was significantly influenced by the interaction between genotype and seasonal variability across most harvests.

Across the different drought intensities, a significant improvement in dry matter yield by genotypic variation were observed from phase 1 to phase 4, indicating that alfalfa yield is not genetically fixed but exhibits substantial phenotypic plasticity in response to seasonal conditions. This finding is consistent with the well-documented complexity of breeding for forage yield, where genotypic performance is strongly environmental specific (Annicchiarico et al., 2016; Valliyodan et al., 2016; He et al., 2022). In contrast, phase 3 did not show significant differences, suggesting that environmental conditions during this period reduced variability among genotypes and years. Across all seasons and phases, variegated alfalfa consistently produced higher dry matter yield than Hunor, highlighting its superior adaptability, regrowth capacity, and yield stability under varying seasonal conditions. These findings support the previous studies stating that varietal selection on yield under drought is mediated by many key traits; specifically, drought-tolerant varieties often possess a deeper and more prolific root system, enhancing water foraging from subsoil layers and directly contributing to yield stability under water deficit (Griffith 1993; El-Ramady et al., 2020). The highest overall yields were generally associated with variegated alfalfa in 2024 and 2025, particularly during phase 2 and phase 3, likely due to favourable climatic conditions and efficient biomass accumulation. Conversely, Hunor had the lowest yields, especially in the later phase, with the lowest value observed in phase 4 of 2025, which may be attributed to cumulative stress effects and reduced persistence. This finding aligns with other studies demonstrating that total dry matter yield in alfalfa varieties decreases significantly under varying environmental conditions and cumulative stress effects (McDonald et al., 2021). Specifically, the observed fluctuations across different harvest phases suggest that varietal selection must account for specific agronomic traits like stem density and heritability to optimize yield under variable weather patterns (Mubarak et al., 2023).

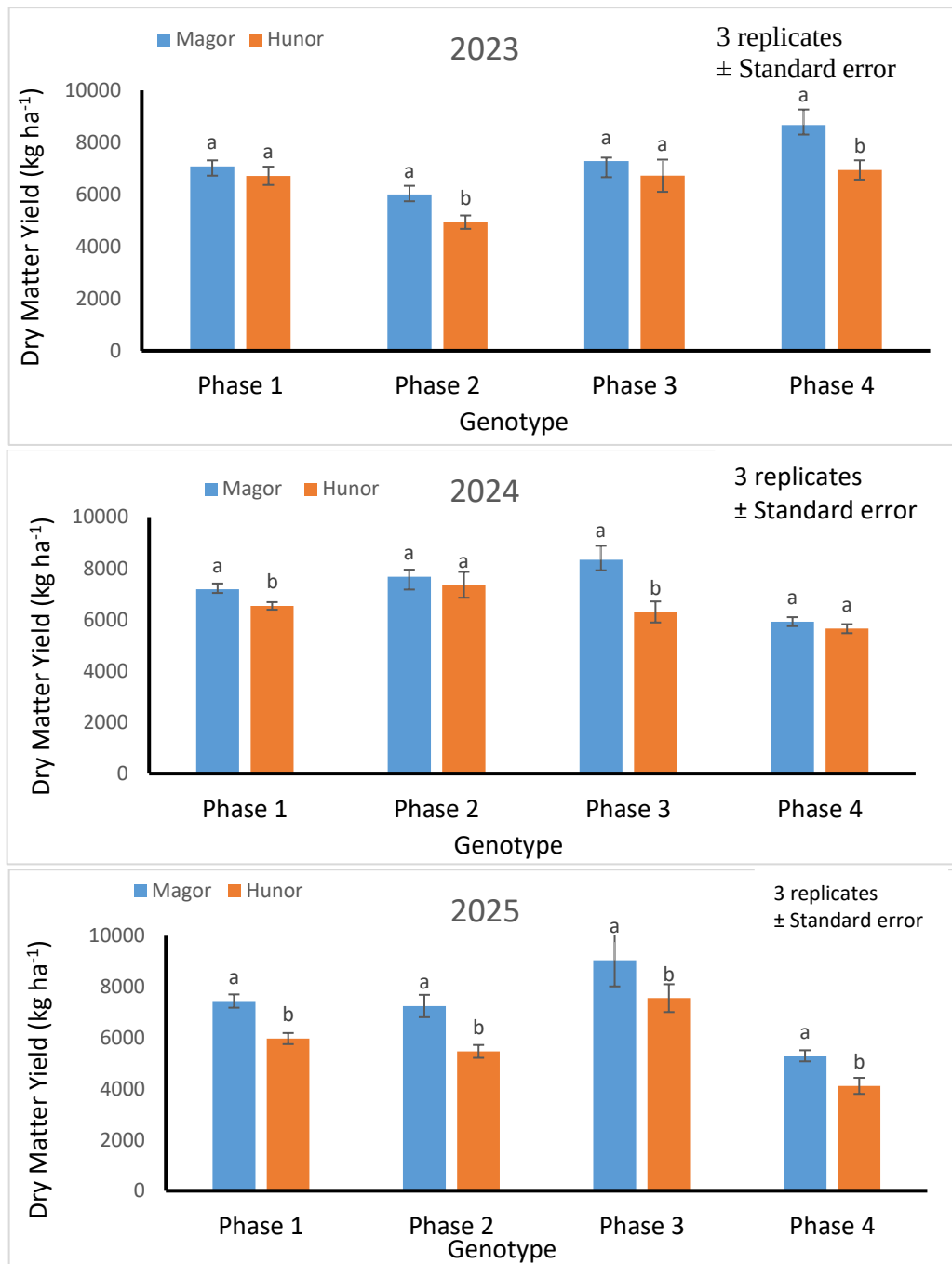


Figure 8: Effect of genotype variability on dry matter yield (kg ha⁻¹) of alfalfa crop during the three cropping seasons (Debrecen, 2023-2025).

*Note: Columns represent annual mean values of treatments (4) and repetitions (3) ± standard error (n = 12). Different letters mean significant differences between genotypes at the same at p = 5%. ** 1st phase: from 1st to 2nd cutting; 2nd phase: from 2nd to 3rd cutting; 3rd phase: from 3rd to 4th cutting, 4th phase: from 4th to 5th cutting.*

Furthermore, significant genetic variation exists for dry matter yield even within similar maturity stages, suggesting that specific varietal selection is crucial for maximizing productivity in temperate environments (Min et al., 2012 Katanski et al., 2020). Researchers have similarly documented significant genotypic differences in herbage dry matter yield among various alfalfa cultivars, with overall mean yields ranging considerably depending on the genetic background and environmental adaptability (Kebede et al., 2017). Overall, variegated alfalfa ‘Magor’ emerges as a more resilient and broadly adaptable genotype, with its falcata ancestry likely conferring buffering capacity against environmental variability. In contrast, ‘Hunor’ behaves as a context-dependent cultivar, performing well under optimal conditions but carrying greater risk in environments prone to late-season or cumulative stress. Under increasing climate uncertainty, sustainable alfalfa production requires selection strategies that prioritize yield stability, exemplified by Magor, alongside maximum yield potential. These findings provide clear guidance for both breeding objectives and cultivar recommendation decisions.

4.2.3 Total dry matter yield under biostimulant treatment application and genotypic variation in two alfalfa genotypes across three cropping seasons (2023-2025)

Total dry matter yield of alfalfa was markedly influenced by biostimulant treatments, genotype, and year under drought stress conditions (**Figure 9**). Across all three experimental years, both genotypes, *Medicago x varia* Martyn (Magor), a variegated alfalfa, and *Medicago sativa* L. (Hunor), exhibited a consistent increase in dry matter yield in response to biostimulant application compared to the untreated control (T0), although the magnitude of response varied between treatments and genotypes.

In 2023, the minimum total dry matter yield was observed in both genotypes, particularly under control conditions (T0), with Magor producing 34,111 kg ha⁻¹ and Hunor also producing 29,176 kg ha⁻¹. However, biostimulant treatment application significantly improved total dry matter yield in both genotypes. The highest total dry matter yield of 37,592 kg ha⁻¹ was observed under T2 in Magor, whereas Hunor showed its maximum total dry matter yield of 35,314 kg ha⁻¹ under T3. Additionally, T1 and T3 treatments also resulted in noticeable yield improvements compared to T0 in both genotypes.

In 2024, the overall total dry matter yield increased substantially across all treatments and both genotypes compared to 2023. However, the minimum dry matter yield was again observed under control plots for both genotypes, with Magor and Hunor producing 35,808

kg ha⁻¹ and 33,024 kg ha⁻¹, respectively. Application of T2 treatment was noted with the highest total dry matter yield for both genotypes, reaching 46,711 kg ha⁻¹ in Magor and 42,828 kg ha⁻¹ in Hunor. Comparatively, T1 and T3 treatments also significantly enhanced yield relative to the control, indicating a strong positive effect of biostimulant application under drought conditions.

In 2025, total dry matter yield declined across all treatments compared to 2024, reflecting stronger drought stress conditions. However, biostimulant treatments continued to mitigate yield losses. T2 consistently produced the highest total dry matter yield of 43,301 kg ha⁻¹, which was observed in Magor, with Hunor also showing a comparatively moderate response, with the highest yield of 32,551 kg ha⁻¹ observed under T2, whereas the lowest total dry matter yields of 29,045 kg ha⁻¹ and 24,935 kg ha⁻¹ were observed under T0. T1 and T3 also improved the yield relative to the control in both genotypes. Across all experimental years and treatments, *Medicago x varia* Martyn (Magor) consistently produced higher total dry matter yield than *Medicago sativa* (Hunor). This trend was evident under both control and biostimulant-treated plots, indicating a stronger inherent drought tolerance or better responsiveness to biostimulant application in Magor. The yield advantage of Magor over Hunor was particularly pronounced under the T2 treatment across all three years. Despite lower absolute yields, Hunor showed a positive and stable response to biostimulant treatments, especially under T1, T2, and T3, demonstrating its capacity to benefit from biostimulant application under abiotic stress. Overall, the study demonstrates that biostimulant treatment application substantially enhanced the total dry matter yield of alfalfa under drought stress, with consistent positive effects observed across years and genotypes. Among the treatments applied, T2 proved to be the most effective, outperforming the remaining treatments (T0, T1 and T3), producing the highest total dry matter yield in both genotypes across all experimental years (2023-2025), with overall total dry matter yield increased by 27% over untreated plots whereas T3 and T1 also improved yield by 23% and 18% compared to the control. This indicates a strong capacity of T2, T3, and T1 to mitigate drought-induced yield losses, likely through improved physiological resilience, enhanced nutrient uptake, or stimulation of stress-adaptive metabolic pathways. These findings align with previous reports showing that biostimulants improve biomass accumulation in legumes under water-limited conditions by enhancing photosynthetic efficiency, root growth, and antioxidant activity (Bulgari et al., 2015; Sible et al., 2021; De Beer et al., 2023; El Sabagh et al., 2025). Similarly, some studies highlighted that, under suboptimal soils,

inoculation of beneficial bacteria (*Bacillus* spp.) and fungi (*Arbuscular mycorrhizae*) enhances yield by improving soil nutrient availability and plant nutrient status (Cao et al., 2023; Li et al., 2024). Furthermore, the 27%, 23% and 18% yield increments under T2, T3 and T1 across the study period align with findings from a three-year trial by Marinova et al. (2023) who reported a 5% rise in alfalfa dry matter yield with a vermicompost-derived biostimulant, indicating improved resilience and productivity over multiple seasons, whereas a 30% increase in dry matter yield was reported under a seaweed-containing biostimulant in other forage crops (Akdag et al., 2024). However, the magnitude and consistency of the response observed in this study, particularly across contrasting climatic years, suggest that the treatments (T1, T2 and T3) may provide a robust buffering effect against drought stress.

Medicago x varia Martyn (Magor) consistently outperformed Hunor in terms of total dry matter yield under both control and biostimulant-treated conditions. This superior performance indicates a stronger inherent drought tolerance or a better responsiveness to biostimulant treatment application in Magor. The pronounced yield advantage of Magor under T2 treatment suggests a positive genotype x biostimulant interaction, where the genetic background enhances the effectiveness of external stress-mitigating inputs. Such genotype-dependent responses to biostimulants have been reported in forage legumes and other crops, where differences in root architecture, stomatal regulation, or osmotic adjustment influence biomass production under drought (Abdali et al., 2023). In contrast, although Hunor exhibited lower absolute yields, its consistent yield improvement under biostimulant treatments highlight its capacity to benefit from external stress-ameliorating strategies.

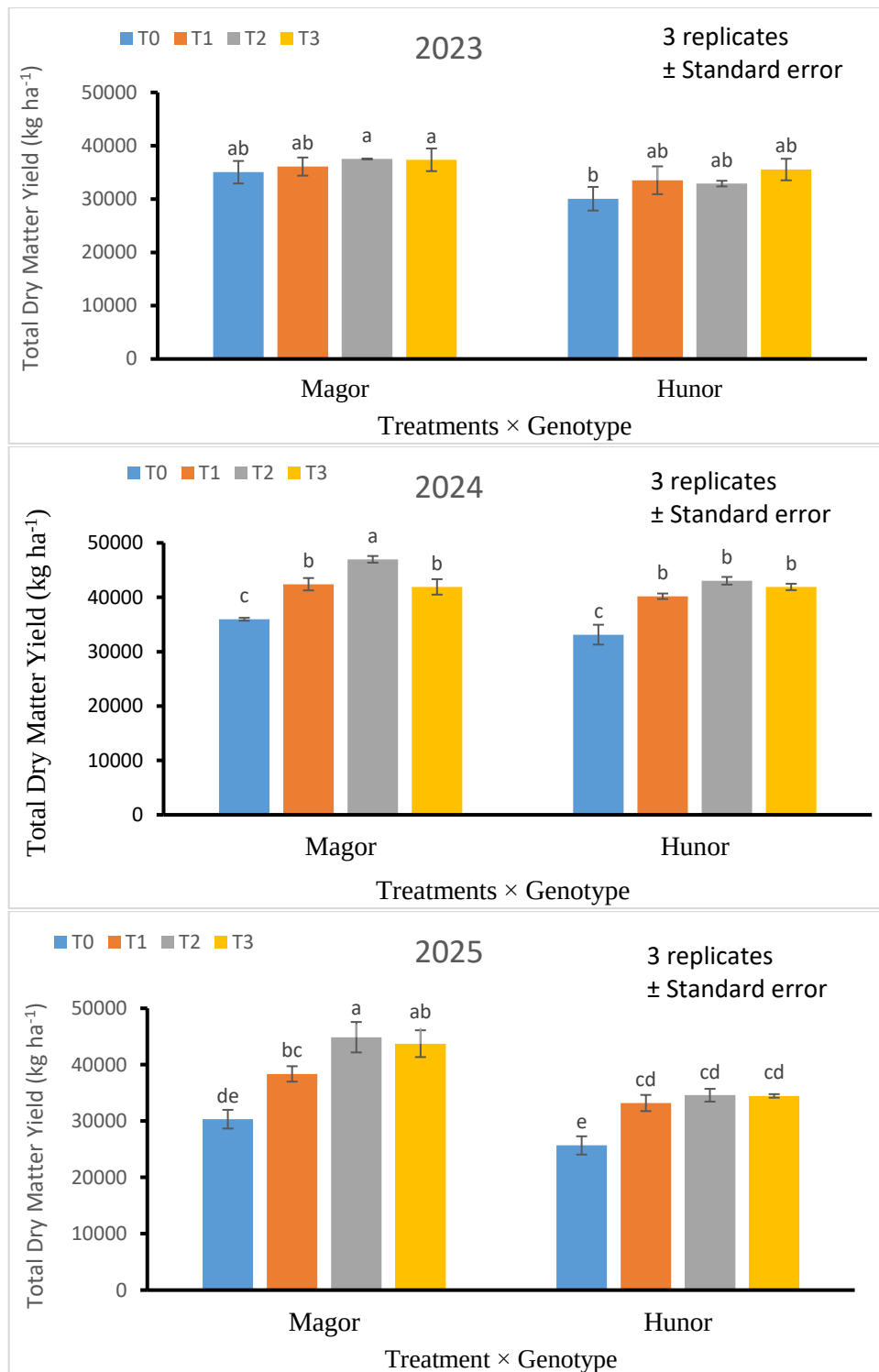


Figure 9: Effect of biostimulant treatments and genotype variability on total dry matter yield (kg ha^{-1}) of alfalfa crop during the three cropping seasons (Debrecen, 2023-2025).
Note: Columns represent annual mean values of treatments (4), genotype (2) and repetitions (3) $\pm$ standard error ($n = 24$). Different letters mean significant differences between treatments at $p = 5\%$.

4.2.4 Interactive effects of biostimulant treatments and environmental conditions on alfalfa total dry matter yield across three cropping seasons (2023-2025)

The combined evaluation reveals a significant ($p < 0.05$) effect of biostimulant treatment x environment (two-way interaction) and genotype variation x environment; however, no significant effect was detected for biostimulant x genotype variation or biostimulant x genotype variation x environment (B x G x E) three-way interactions on total dry matter yield. The **(Figure 10)** below illustrates the summary of the biostimulant treatment x environment observed total dry matter yield in alfalfa, which indicates that the effect of biostimulant application differed between the three cropping seasons. Generally, the total dry yield of alfalfa significantly increased progressively across the three cropping seasons. In the 2023 cropping season, biostimulant-treated plots produced higher yields than the untreated control, with the highest increase of 13.5% observed under T3, followed by 11% and 10% under T2 and T1, respectively. Similarly, in the 2024 cropping season, plots treated with biostimulants significantly increased total dry matter yield, with the highest increase of 30% observed at T2, followed by T3 with 20.7% and T1 with 19.2% compared to the control, respectively. In the 2025 cropping season, plots treated with biostimulant showed further enhancements in total dry matter yield, with the greatest response observed in T2, which consistently produced the highest yields of 40.5%, followed by T3 with 37% and T1 with 25% when compared to the untreated plots. Overall, the greatest response at T2 in the 2024 and 2025 cropping seasons suggests that environmental conditions during these seasons were especially conducive to biostimulant efficacy. In contrast, while yield increases were also recorded in 2023 cropping season, the differences among treatments were less pronounced, and the overall response magnitude was smaller compared to the preceding cropping seasons. The progressive increase in total dry matter across the three cropping seasons highlights that the applied treatments enhanced alfalfa growth and development under varying environmental conditions. This finding aligns with previous studies showing that biostimulants stimulate root growth, leading to improvements in biomass accumulation, nutrient uptake, and metabolic efficiency (Calvo et al., 2014; Du Jardin, 2015). However, the greatest response observed in the 2025 and 2024 cropping seasons, particularly under T2, highlights the significance of biostimulant x environment interaction.

Several studies have shown that biostimulants often exhibit their greatest efficacy under moderate abiotic stress, where they enhance physiological resilience through mechanisms such as improved osmotic adjustment, antioxidant activity, and hormonal regulation

(Rouphael and Colla, 2020; Bulgari et al., 2019). The greatest pronounced total dry matter yield at T2 (40%) in 2025 and 30% in 2024 suggests that environmental conditions during these seasons, potentially involving moderate drought, created a medium in which the biostimulant could express its full functional potential. This finding is consistent with previous studies reporting that biostimulant effects are amplified when plants experience suboptimal growing conditions (Yakhin et al., 2017). In contrast, the smaller treatment differences observed in 2023 may reflect more favourable baseline conditions, where the crop relied less on biostimulant-mediated stress mitigation. Under such circumstances, yield responses tend to be modest because plants are already performing near their physiological optimum (du Jardin, 2015). The variability between years therefore underscores the context-dependent nature of biostimulant performance. Specifically, seasonal variations in temperature and precipitation strongly influence biostimulant effectiveness, either mitigating or exacerbating responses (Godlewska and Ciepiela, 2018; Franzoni et al., 2023).

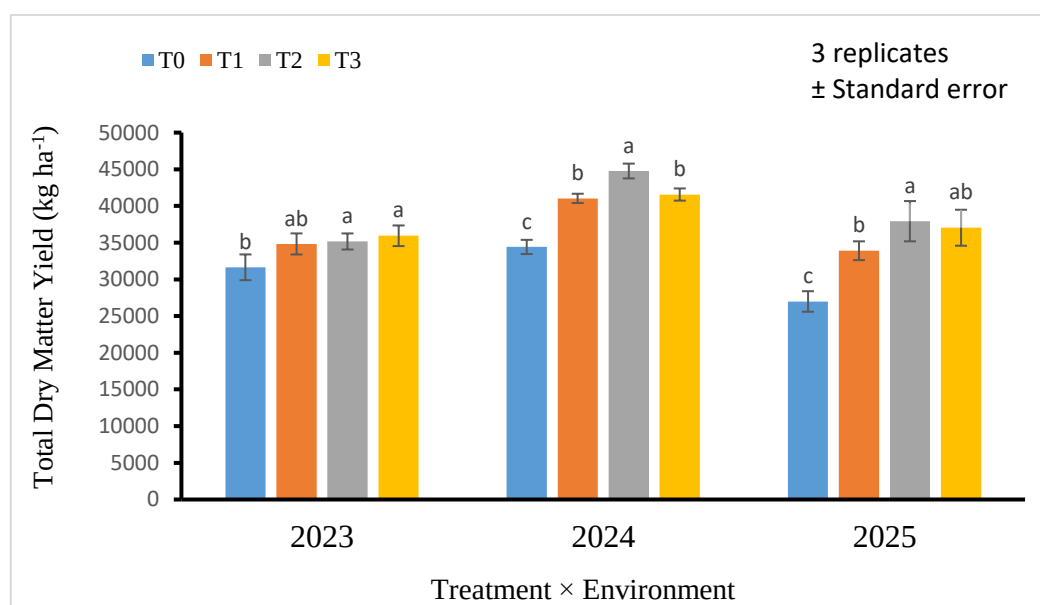


Figure 10: Interactive effect of biostimulant treatment x environment (B x E) on total dry matter yield of alfalfa crop cultivated over a three-year period (Debrecen, 2023 – 2025). *Note: Columns represent annual mean values of genotypes (2), and repetitions (3) $\pm$ standard error ($n = 6$). Different letters mean significant differences between treatments at $p = 5\%$.*

4.2.5 Interactive effects of genotypic divergence and environmental condition on alfalfa total dry matter yield across three cropping seasons (2023-2025)

A highly significant genotype x year interaction was observed for total dry matter yield (TDMY), indicating that genotypes differed in their yield stability across the three

cropping seasons. In the 2023 cropping season, Magor genotypes exhibited high productivity, with yields 10.1% higher than those of Hunor genotypes (**Figure 11**). Similarly, in the 2024 cropping season, Magor maintained its superior performance by producing 5% more total dry matter yield relative to Hunor. In the 2025 cropping season, this advantage was even more pronounced in Magor, with a significant 25% increase over Hunor (**Figure 11**). Although Magor significantly outperformed Hunor, the latter also delivered strong performance across the three seasons, reflecting a positive response to the applied treatments. Overall, Magor showed the greatest response, yielding dry matter gains ranging from 5% to 25% across seasons and demonstrating broad adaptability and stable performance across environments.

The observed significant $G \times E$ interaction highlights how environmental conditions influenced the magnitude of yield expression, a common phenomenon in forage and field crop evaluations (Kebede et al., 2017; Watt et al., 2021; Behera et al., 2024). However, Magor's consistent superiority over Hunor across seasons indicates a non-crossover interaction, where genotype rankings remain unchanged across environments. Such non-crossover interactions are particularly valuable in breeding and variety recommendation, as they simplify selection decisions (Pour-Aboughadareh et al., 2025). Furthermore, Magor's stable performance suggests it possesses traits conferring resilience under varying seasonal conditions, such as efficient biomass partitioning, superior stress tolerance, or enhanced resource-use efficiency factors known to support stable dry matter production. This consistent yield advantage aligns with findings from similar studies, where certain genotypes maintained their performance despite environmental variability (Edwards et al., 2016; Luo et al., 2020). Moreover, given its reliable yield and stability, Magor is a strong candidate for recommendation in similar environments. Stable alfalfa genotypes are especially preferred in smallholder and variable production systems prone to environmental unpredictability (Kebede et al., 2017; Manyasa et al., 2017; Mubarak et al., 2023; Zheng et al., 2023). These results thus support adopting Magor as a reliable, high-yielding option for farmers seeking consistent dry matter production. This stability is particularly crucial for maintaining forage availability during critical periods, as selecting productive genotypes is essential for the viability of animal production systems (Stida et al., 2018).

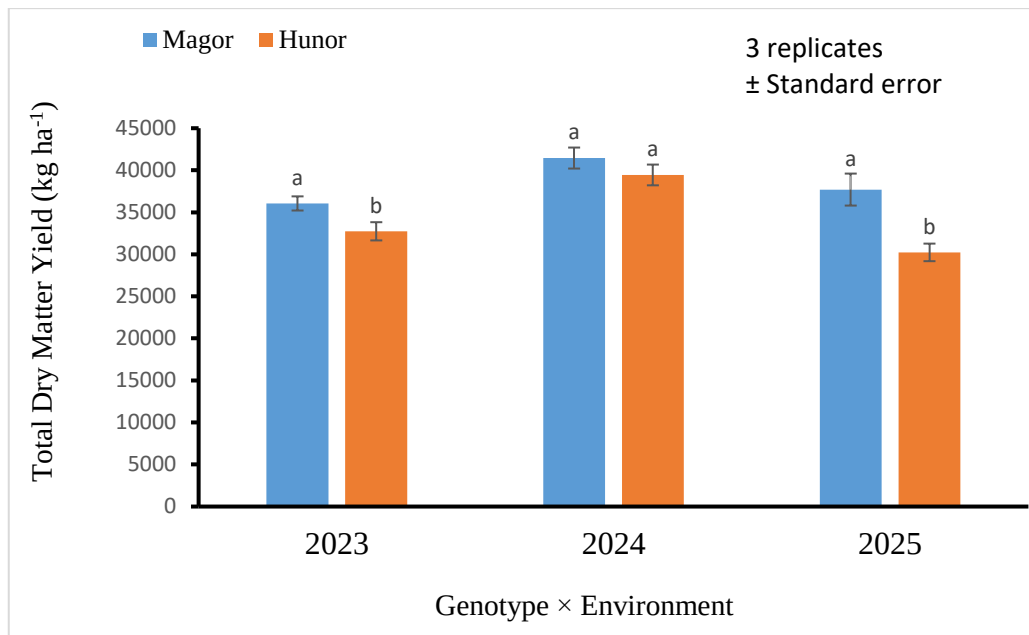


Figure 11: Interactive effect of genotypic variation x environment (B x E) on total dry matter yield of alfalfa crop cultivated over a three-year period (2023–2025).

Note: Columns represent annual mean values of biostimulant treatment (4) and repetitions (3) $\pm$ standard error ($n = 12$). Different letters mean significant differences between treatments at $p = 5\%$.

4.2.6 Complex evaluation of biostimulant treatment, genotype, year and their interactions on total dry matter yield alfalfa under contrasting drought (2023-2025)

A highly significant effects of treatment, genotype, year, treatment x year, and genotype x year interactions were observed, indicating a very large effect of biostimulant treatment on total dry matter yield (partial $\eta^2 = 0.69$), as did genotype (partial $\eta^2 = 0.49$) and year (partial $\eta^2 = 0.66$) (**Table 11**). The treatment x genotype interaction showed a moderate effect size (partial $\eta^2 = 0.09$), whereas the treatment x year and genotype x year interactions were large (partial $\eta^2 = 0.27$ and 0.22). The three-way interaction between treatment x variety x year also exhibited a large effect size (partial $\eta^2 = 0.14$), although it was not statistically significant. These findings show that biostimulant treatments, genotype, and crop year accounted for the greatest variation in alfalfa total dry matter yield; however, genotype-specific responses to treatments are strongly modified by crop year variation, suggesting that environmental influences are vital. This highlights the necessity of considering genotype-by-environment interactions when optimizing alfalfa cultivation practices, as differing environmental conditions can significantly alter the efficacy of specific biostimulant treatments and genotypic performance (El-Hifny et al., 2019). Other studies also demonstrated that while certain biostimulants may enhance yields, their effectiveness depends on both the specific alfalfa genotype and prevailing

environmental conditions, particularly annual precipitation (Godlewska and Ciepiela, 2018). This aligns with previous research emphasizing the influence of environmental factors, such as rainfall differences, on plant performance and the differential effectiveness of treatments and genotypes (Yousefi et al., 2025).

Table 11: Complex evaluation of biostimulant treatment, genotype, year and their interactions on total dry matter yield of alfalfa (Debrecen, 2023–2025).

Source of variation	DF	F	P	Partial η^2
Treatment	3	33.87	<.001	0.69
Genotype	1	45.7	<.001	0.49
Year (Environment)	2	43.99	<.001	0.66
Treatment x Genotype	3	1.5	0.227	0.09
Treatment x Year	6	2.77	0.022	0.27
Genotype x Year	2	6.81	0.003	0.22
Treatment x Genotype x Year	6	1.26	0.294	0.14

***df = degree of freedom, F = F-value, P = significance, partial η^2 = partial eta square.*

4.2.7 Correlation analysis of sum of precipitation, PaDI value, growth, physiological parameters, dry matter yield of alfalfa cultivated under contrasting drought conditions (2023-2025)

The correlation analysis revealed significant ($p < 0.001$) relationships among Sum of precipitation, PaDI value, total dry matter yield (TDMY), growth (PH), normalised difference vegetation index (NDVI) and net photosynthetic rate (NPR) of alfalfa measured across three cropping seasons, with results summarized in **Table 12**. The analysis showed strong positive correlations between total dry matter yield, growth traits, and vegetative indices, whereas negative correlations existed between total dry matter yield and gas exchange traits. For instance, total dry matter yield exhibited strong positive correlations with plant height, particularly height at 21 DAH ($r = 0.639$, $p < 0.001$) and plant height at 28 DAH ($r = 0.553$, $p < 0.001$), indicating that late vegetative growth plays a critical role in final biomass accumulation. Plants with adequate height likely possess greater leaf area and improved light interception, enhancing cumulative carbon assimilation and biomass yield. Similar positive associations between plant height and forage yield in alfalfa have been reported by Fang et al. (2024) who observed that plant stature is a reliable indicator of forage productivity in perennial legumes. Other studies indicate that morphological traits such as height and stem density strongly influence perennial forage crop biomass production under water-limited environments (Annicchiarico et al., 2015). A moderate positive correlation was also observed between

total dry matter yield and plant height at 14 DAH ($r = 0.225$), suggesting that early vegetative growth alone is insufficient to predict final biomass, particularly under drought where growth dynamics may shift later in the season. Drought stress often affects growth rate, canopy expansion, and carbon allocation patterns over time (Chaves et al., 2003; Edwards et al., 2016). Thus, later-stage morphological development appears to be a better indicator of yield potential.

The drought index exhibited consistent negative correlations with plant height at 14 days after harvest, NDVI from 14 to 28 DAH, and total dry matter yield, as indicated by negative values ranging from (-0.071 to -0.819). This pattern across these traits indicates that the drought index effectively captured the severity of water deficit conditions in the experimental area, confirming that NDVI serves as a sensitive remote-sensing indicator of drought-induced canopy decline, early plant height reflects immediate growth suppression under water deficit, and dry matter yield represents the cumulative impact of drought on productivity. These findings align with studies demonstrating that drought stress reduces canopy greenness, vegetative growth, and biomass production in alfalfa and other forage crops (Kulkarni et al., 2018; Liu et al., 2018; Kačienė et al., 2022).

Vegetative indices, especially NDVI at later measurements (21 and 28 DAH), also exhibited positive correlations with total dry matter yield ($r = 0.603$) and ($r = 0.638$) respectively. As NDVI is widely recognized as a proxy for canopy greenness, chlorophyll content, and leaf area index, the strong association between NDVI and biomass production aligns with findings from previous studies reporting that NDVI is strongly linked to crop biomass and productivity across several crops (Meng et al., 2013). In forage systems, NDVI has been successfully used to estimate alfalfa biomass and canopy development under variable moisture conditions (Koc et al., 2023).

In contrast, moderate negative correlations were observed between total dry matter yield and NPR at both early and late measurements (14 and 28 DAH). Although photosynthesis is fundamentally responsible for carbon assimilation, instantaneous gas exchange measurements often do not directly translate to final yield, as biomass reflects integrated activity over time rather than single-point measurements. Under drought, plants may exhibit physiological adjustments such as stomatal regulation, osmotic adjustment, or altered carbon allocation that sustain photosynthesis temporarily but limit long-term growth (Flexas et al., 2002), explaining the negative association between NPR and biomass yield. In addition, drought-stressed plants maintain higher leaf-level photosynthesis due to reduced canopy size, which minimizes self-shading and increases

light per leaf area; however, the smaller canopy limits total carbon fixation, resulting in lower biomass. Similar decoupling between instantaneous photosynthesis and yield has been reported in drought-stressed legumes and cereals (Xu et al., 2009).

Inter-trait correlations also revealed consistent patterns. Strong positive correlations were observed among plant height variables across growth stages: heights at 21 and 28 DAH were highly correlated ($r = 0.904$, $p < 0.001$), while height at 14 DAH showed moderate positive associations with later measurements, indicating stable vertical growth throughout the vegetative phase ($r = 0.512$ and 0.396). This suggests that genotypes or treatments promoting early canopy establishment maintain growth advantages later, contributing to higher biomass.

Vegetation indices were strongly intercorrelated, particularly NDVI at 21 and 28 DAH ($r = 0.885$, $P < 0.001$), highlighting their sensitivity to canopy greenness and biomass accumulation. These results indicate that NDVI effectively captures temporal changes in canopy development and biomass. Previous research has shown that NDVI during mid-vegetative stages is a reliable predictor of forage biomass (Jenal et al., 2021)

NDVI variables also showed moderate to strong positive correlations with plant height with r value ranging from $0.308 - 0.418$, indicating that increased canopy vigor is linked to improved growth. Taller plants produce denser canopies with higher chlorophyll content, leading to stronger NDVI signals.

NPR displayed moderate associations with vegetation indices. NPR at 14 and 28 DAH negatively correlated with all NDVI values, with the strongest negative association between NPR and NDVI at 14 DAH ($r = -0.755$). These findings suggest that dense canopies reduce leaf-level photosynthetic efficiency due to shading. Self-shading limits light to lower leaves, reducing photosynthetic rates despite increased overall biomass. Similar effects have been reported in dense forage stands and cereal crops (Posada et al., 2012). Overall, morphological traits and canopy development (plant height and NDVI) are stronger determinants of final biomass yield than instantaneous physiological measurements under drought. While NPR reflects short-term stress responses, growth traits integrate performance over time and thus correlate more strongly with dry matter yield.

Table 12: Correlation among precipitation sum for period, Pálfai drought index, growth, physiological parameters and dry matter yield of alfalfa under contrasting drought conditions (Debrecen, 2023–2025)

	P03-07	PaDI	PH_at14DAH	PH_at21DAH	PH_at28DAH	NDVI_at14DAH	NDVI_at21DAH	NDVI_at28DAH	NPR_at14DAH	NPR_at28DAH	TDMY
P03-07	-										
PaDI	-0.960	-									
PH_at14DAH	0.287	-0.198	-								
PH_at21DAH	-0.266	0.249	0.512	-							
PH_at28DAH	-0.479	0.489	0.396	0.904	-						
NDVI_at14DAH	0.928	-0.819	0.418	-0.141	-0.307	-					
NDVI_at21DAH	0.075	-0.118	0.436	0.728	0.589	0.099	-				
NDVI_at28DAH	0.302	-0.402	0.308	0.559	0.352	0.237	0.885	-			
NPR_at14DAH	-0.896	0.932	-0.163	0.259	0.492	-0.755	-0.116	-0.374	-		
NPR_at28DAH	-0.409	0.483	-0.156	0.119	0.225	-0.291	-0.177	-0.291	0.543	-	
TDMY	-0.075	-0.071	0.225	0.639	0.553	-0.102	0.608	0.638	-0.049	-0.203	-

Note: P03–07: precipitation sum for period March–July; PaDI: Pálfai Drought Index value, PH = plant height, NDVI = normalised difference vegetation index, NPR = net photosynthetic rate, TDMY = total dry matter yield, DAH = day after harvest

4.3 Effect of Biostimulants Treatments, Genotype Variability and Environment on Quality Parameters of *Medicago sativa* L. and *Medicago x varia* Martyn

4.3.1 Differential response of protein content in *Medicago sativa* L. and *Medicago x varia* Martyn to biostimulant treatments and genotype variation across three cropping seasons (2023-2025)

Prior to determining the alfalfa quality parameters from the laboratory, plant samples were collected only from the second harvest in each experiment due to the high financial cost involved. Protein content was highly significantly ($p < 0.001$) affected by the biostimulant treatment, genotype variation, and the biostimulant $\times$ genotype variation interaction only in the 2024 cropping season. In the 2024 cropping season protein content was significantly improved under the interaction between the biostimulant treatments and genotype variations, demonstrating that both genotypes (Magor and Hunor) responded differently to biostimulant treatment application. In the Magor genotype, protein content increased under the application of biostimulant treatments, reaching the maximum improvement of 12% at T3, 10% at T2 and 9% at T1 compared to the control (T0) (**Figure 12**). The Hunor genotype also showed a positive response, with protein content increases ranging from 8% at T2 to 11% at T1. These contrasting response trends confirm that the effectiveness of these biostimulant treatments is cultivar-dependent. Meanwhile, in the 2023 and 2025 cropping seasons, the treatment, genotype variation and their interaction were all not significant ($p > 0.05$). In the 2023 cropping season, though not significantly different, protein content in Magor was improved, ranging from 0.1% at T2 to 1.8% at T3 compared to the control (T0). However, a slight decline in protein content was observed in Hunor ranging from -2% at T2 to -3.4% at T3 (**Table 13**). A similar trend was observed in the 2025 cropping season, where protein content in Magor increased from 0.7% at T2 to 3.1% at T1 whereas in Hunor, protein decreased by -0.7% at T1 and increased at T2 by 2.8% compared to the control (T0). Overall, across the three cropping seasons, significant effects of biostimulant treatment application, genotype variation and their interaction were observed only in 2024, whereas 2023 and 2025 showed no measurable response. This highlights a strong year-to-year variability aligned with previous reports indicating that biostimulant efficacy is highly dependent on environmental conditions. Many studies have demonstrated that biostimulants tend to improve crop protein content and yield benefits under moderate stress conditions, particularly drought, salinity, or imbalanced nutrient conditions, when plants can physiologically respond to applied compounds (Goñi et al., 2018; Franzoni et al., 2022; Rezaei-Chiyaneh et al., 2023). This significant response observed in 2024 suggests that

the environmental condition during that season created a similar stress-responsive context, enabling both genotype variation and biostimulant treatment effects to be expressed. In contrast, the lack of significant effects in 2023 and 2025 is consistent with the findings of (Cinantya et al., 2024), who reported no effect of biostimulant under extremely unfavourable cropping conditions. These findings reinforce the notion that biostimulant performance is not universal but strongly modulated by seasonal conditions, supporting that broader conclusion that multiyear evaluations are essential for accurately assessing treatment consistency and genotype responsiveness.

Table 13: Effect of biostimulant treatment x genotype on protein content of alfalfa crop cultivated during the 2023 and 2025 cropping season (Debrecen, 2023 and 2025)

Treat- ments	2023			2025		
	Magor	Hunor	Mean	Magor	Hunor	Mean
T0	17.11±0.57	17.63 ±0.31	17.37 ±0.44	19.33 ±0.18	19.20 ±0.75	19.26 ±0.46
T1	17.35 ±0.52	17.20 ±0.55	17.27 ±0.54	19.93 ±0.53	19.07 ±0.46	19.50 ±0.50
T2	17.12 ±0.46	17.27 ±0.82	17.19 ±0.64	19.47 ±0.95	19.73 ±0.40	19.60 ±0.68
T3	17.41 ±0.44	17.03 ±0.20	17.22 ±0.32	19.63 ±0.89	19.47 ±0.67	19.55 ±0.78
Means	17.24 ±0.50	17.28 ±0.47		19.59 ±0.64	19.36 ±0.57	

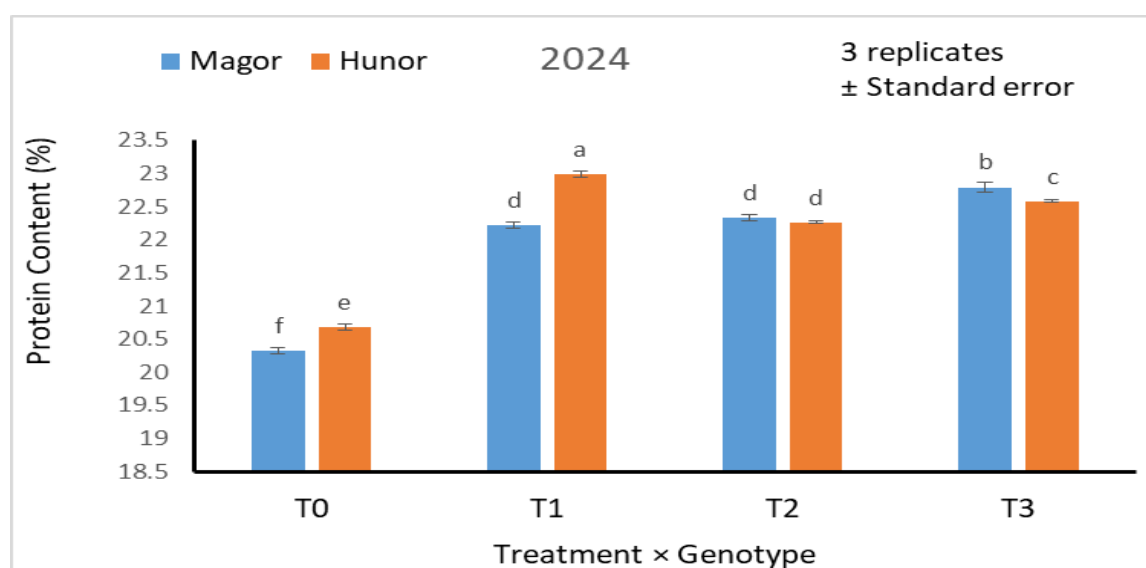


Figure 12: Interactive effect of biostimulant treatment x genotype (B x G) on protein content of alfalfa crop cultivated during the 2024 cropping season (Debrecen, 2024).

Note: Columns represent annual mean values of genotypes (2) and repetitions (3) ± standard error (n = 6). Different letters mean significant differences between treatments at p = 5%.

4.3.2 Interactive effects of biostimulant treatments and environmental conditions on protein content of alfalfa across three cropping seasons (2023-2025)

Based on the combined evaluation, analysis of variance showed no significant ($p > 0.05$) effects of genotype variation x environment, biostimulant x genotype variation, or

biostimulant x genotype variation x environment (B x G x E) interactions. However, a significant ($p < 0.05$) biostimulant treatment x environment interaction was detected, indicating that the performance of these treatments was strongly influenced by environmental conditions, with responses varying across experimental years. **Figure 13** below illustrates the effectiveness of biostimulants across varying experimental years.

In 2024, protein content increased progressively with biostimulant treatments, with T3 showing the highest value, followed by T1 and T2, while the control had the lowest. This reflects a significant increase of protein content by 11%, 10% and 8.7% under T3, T1 and T2, respectively, over the control (T0). In 2025, however, the response was less pronounced than in 2024; biostimulant treatments increased protein content by 2% and 1.4% compared to the control, and T1 did not differ significantly from T3. In 2023, protein content was much lower than in 2024 and 2025, declining by -1% under T2 and by -0.8% and -0.5% under T3 and T1, respectively, which highlights the negative effects of these treatments on alfalfa protein content. Overall, the magnitude of the biostimulant treatments' (T1, T2 and T3) effects on protein content was greater in 2024 than in 2025 and 2023; however, the differences between these treatments were statistically similar. This confirms the significant biostimulant treatment x environment interaction for protein content, indicating that the effectiveness of these treatments depended on seasonal conditions. This environmental modulation of treatment efficacy aligns with previous research demonstrating that crude protein rates in alfalfa vary significantly under different environmental conditions (Turan, 2019). In 2024, the clear, treatment-dependent increase in protein content with T3 suggests that the product enhanced nitrogen assimilation or protein synthesis under prevailing stress conditions. A similar observation has been made by (Fu et al., 2018; Kaciene et al., 2022). In contrast, the weaker response in 2023, particularly the difference among treatments (T1, T2 and T3), may reflect more favourable growing conditions, where the addition of biostimulant inputs did not translate into further protein accumulation. A similar year-dependent response of biostimulants has been reported in field studies where environmental variability modulated their efficacy (Fusco et al., 2025). However, the inconsistency in treatment efficacy across years underscores the complexity of biostimulant action, as effects can differ between crop species and are influenced by specific environmental factors such as soil pollution and climate change (Baltazar et al., 2021). The variable impact of biostimulants on chemical composition is further complicated by the fact that their effects are difficult to interpret and depend on multiple interacting factors (Boutahiri et al., 2024). For instance, titanium-

based biostimulants have been shown to significantly increase protein compound content in sand lucerne compared to control plants, while sea algae extracts produced statistically insignificant increases despite reported benefits in other studies (Godlewska and Ciepiela, 2018). These findings highlight that the application of these biostimulant treatments is not universally superior and that their benefits on alfalfa protein content are strongly influenced by year-to-year climatic variability. Specifically, temperature and rainfall fluctuations can either mitigate or exacerbate the physiological effects of biostimulant applications on crop quality parameters (Franzoni et al., 2023).

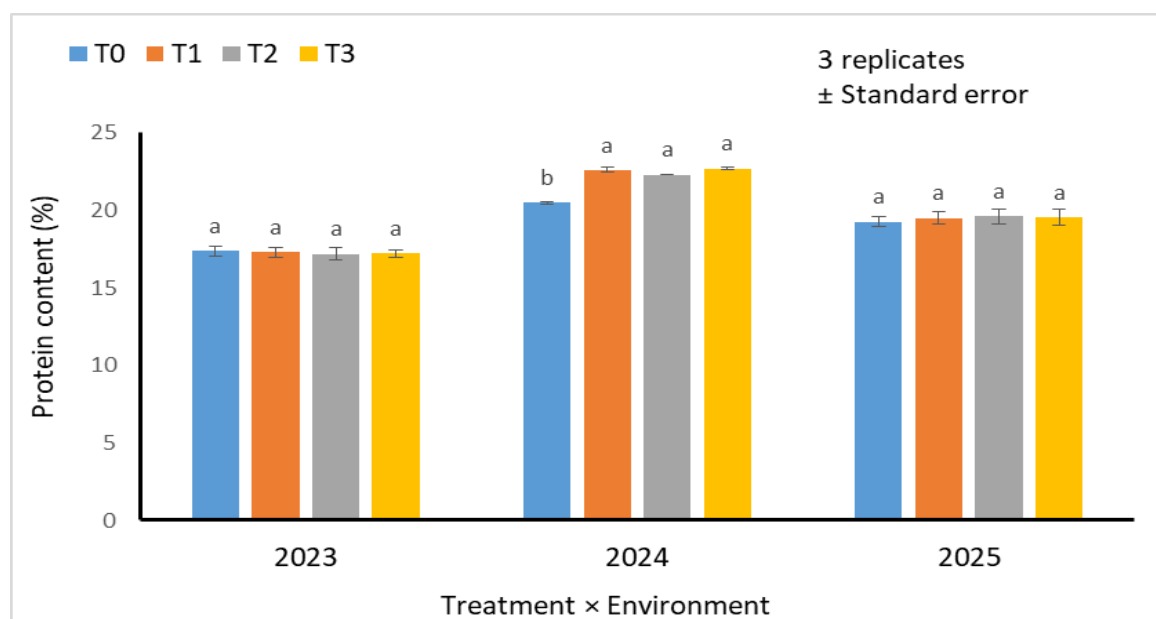


Figure 13: Interactive effect of biostimulant treatment x environment (B x E) on protein content of alfalfa crop cultivated over a three -year period (Debrecen, 2023 - 2025).
Note: Columns represent annual mean values of genotype (2) and repetitions (3) ± standard error (n = 6). Different letters mean significant differences between treatments at p = 5%.

4.3.3 Differential effects of biostimulant treatments and environmental conditions on total sugar content in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023-2025)

The analysis of variance shows that there is no significant ($p > 0.05$) effect of the treatments and genotype variation on the total sugar during the 2023 and 2025 cropping seasons. However, a highly significant ($p < 0.001$) effect was detected by the biostimulant treatments, genotype variation and the biostimulant x genotype variation interaction during the 2024 cropping season. In 2024, total sugar in Hunor significantly increased under the application of biostimulant treatments, reaching a significant enhancement of 11.8% under T1, followed by T3 and T1 with 9.4% and 8.7% compared to the control

(T0) (**Figure 14**). A similar positive enhancement was detected in the Magor genotype, with total sugar increased from 9.4% at T1 to 12.5% at T3 when compared to the control. These results demonstrated that both Hunor and Magor genotypes significantly responded differently to the application of biostimulant under moderate drought stress conditions. In the 2023 cropping season, even though no significant difference existed, total sugar in Magor was improved, ranging from 0.3% at T2 to 2.3% at T3 compared to the control (T0). However, a decline in total sugar was observed in Hunor, ranging from –1.9% at T2 to –2.5% at T1 (**Table 14**).

A similar trend was observed in the 2025 cropping season, where total sugar in Magor increased from 0.8% at T2 to 4.2% at T1 whereas in Hunor, we observed a –0.5% decrease in total sugar at T1 whereas an increase of 4% and 1.3% was observed at T2 and T3, respectively, compared to the control (T0). Overall, the studies show that application of biostimulant treatment significantly increased total sugar, with a significant pronouncement in Magor compared to Hunor. However, the significant increase in total sugar due to the application of biostimulant treatment in both genotypes prevailed only in the 2024 cropping season, contrasted with the absence of effects in the 2023 and 2025 cropping seasons, indicating a strong year-dependent response. Such interannual variability suggests that environmental factors, particularly precipitation patterns and temperature fluctuations, play a critical role in modulating the efficacy of biostimulant applications on carbohydrate metabolism in forage species (Tlahig et al., 2020; Kačienė et al., 2022). Soluble sugar metabolism is highly sensitive to plant water status and thermal conditions, which can either enhance or mask biostimulant-induced metabolic effects, as evidenced by studies where herbicide interactions significantly altered sugar accumulation in soybean (Franzoni et al., 2023). The use of biostimulant are increasingly reported to exert their greatest effects under physiological or environmental stress conditions, especially under moderate stress rather than under optimal or severe limiting conditions (Szparaga et al., 2018; Akdağ et al., 2024). The positive response observed in the 2024 cropping season may therefore reflect environmental conditions that allowed the biostimulant treatments to enhance carbon assimilation or sugar translocation, whereas the lack of response in subsequent years may indicate that environmental conditions were either optimal, negating the need for metabolic adjustment, or too severe, inhibiting the physiological activation required for biostimulant efficacy. This variability aligns with findings that seasonal conditions are strong determinants in defining the effectiveness of several biostimulants, as environmental stressors can either mitigate or exacerbate their

impact on crop quality parameters (Franzoni et al., 2023). Furthermore, the consistent response trend across both genotypes suggests that environmental factors played a more pivotal role than genetic background in determining the magnitude of biostimulant-induced changes in total sugar concentration (Hamade et al., 2024). In perennial leguminous species, carry-over effects from previous seasons can influence the metabolic response and nutrient reserves of plants, potentially explaining the reduced biostimulant effect in the first and third year cropping seasons (Albasri et al., 2024). Overall, these findings highlight that the biostimulant treatments act as a conditional enhancer of sugar accumulation in alfalfa genotypes rather than a stable, year-dependent modifier of carbohydrate metabolism. This conditional nature is supported by research indicating that biostimulant efficacy is highly dependent on crop species and cultivar, with significant variations in non-structural carbohydrate accumulation observed between different grass species (Ciepiela and Godlewska, 2015).

Table 14: Effect of biostimulant treatment x genotype on total sugar of alfalfa crop cultivated during the 2023 and 2025 cropping seasons (Debrecen, 2023 and 2025)

Treatments	2023			2025		
	Magor	Hunor	Mean	Magor	Hunor	Mean
T0	3.41 ±0.10	3.52 ±0.07	3.47 ±0.09	3.85 ±0.05	3.81 ±0.15	3.83 ±0.10
T1	3.47 ±0.11	3.43 ±0.11	3.45 ±0.11	4.01 ±0.13	3.79 ±0.10	3.90 ±0.12
T2	3.40 ±0.08	3.45 ±0.15	3.43 ±0.12	3.88 ±0.16	3.96 ±0.09	3.92 ±0.13
T3	3.49 ±0.09	3.44 ±0.04	3.46 ±0.06	3.90 ±0.19	3.86 ±0.13	3.88 ±0.16
Means	2.95 ±0.09	3.17 ±0.09		3.91 ±0.13	3.85 ±0.12	



Figure 14: Interactive effect of biostimulant treatment x genotype (B x G) on total sugar of alfalfa crop cultivated during the 2024 cropping season (Debrecen, 2024).

Note: Columns represent annual mean values of biostimulant treatment (4) and repetitions (3) $\pm$ standard error ($n = 12$). Different letters mean significant differences between treatments at $p = 5\%$

4.3.4 Interactive effects of biostimulant treatment x environmental conditions on total sugar in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023-2025)

The combined evaluation reveals no significant ($p > 0.05$) effects of genotype variation x environment, biostimulant x genotype variation, or biostimulant x genotype variation x environment (B x G x E) interactions. However, a significant ($p < 0.05$) biostimulant treatment x environment interaction was detected, indicating that the performance of these treatments was strongly influenced by environmental conditions, with responses varying across experimental years. **Figure 15** below illustrates the effectiveness of biostimulants across varying experimental years. In 2024, total sugar significantly increased with biostimulant treatments, with T3 and T1 showing the highest values of 4.53% and 4.52%, followed by T2 with 4.4%, whereas the control had the lowest value of 4.08%. These results reflect a 10.9%, 10.6%, and 9.3% improvement in total sugar under T3, T1 and T2, respectively, compared to the control (T0). In 2025, the response of these treatments was less pronounced compared to the 2024 cropping season. However, there was a marginal increase in total sugar by 2.4%, 1.8%, and 1.3% observed under T2, T1, and T3 compared to the control (T0). In 2023, total sugar was much lower than in 2024 and 2025; in fact, total sugar declined by -1.1% under T2 and -0.43% and -0.17% under T1 and T2, respectively, which highlights the negative effects of these treatments

on alfalfa total sugar. Overall, the magnitude of the biostimulant treatments' effects on total sugar was significantly greater in 2024 (increases of 10.9%, 10.6%, and 9.3% for T3, T1, and T2, respectively, over T0) than in 2025 (marginal increases of 2.4%, 1.8%, and 1.3%) or 2023 (declines of up to -1.1%). This confirms the significant biostimulant treatment x environment interaction for total sugar, indicating that the effectiveness of these treatments depended on environmental conditions. In comparing the individual cropping seasons, we observed that total sugar in 2024 significantly increased by 27.4% and 13.4% over the 2023 and 2025 cropping seasons, whereas 2025 also saw an increase of 12% over the 2023 cropping season. This temporal variation in efficacy observed across the 2024 and 2025 cropping seasons could be due to climatic conditions favourable for the biostimulants to enhance metabolic activity and nutrient uptake, as evidenced by studies showing that biostimulant benefits become more apparent later in the growth cycle when environmental factors support optimal physiological functioning (Godlewska and Ciepiela, 2018; Appiah et al., 2024a). Specifically, applications of biostimulants like Algex and Tytanit have significantly increased monosaccharide content in lucerne irrespective of nitrogen regime, cuts, or study years (Godlewska and Ciepiela, 2018), reinforcing their potential under moderate conditions to boost soluble sugars. Conversely, under less favourable conditions, conditions like the 2023 cropping season marked by treatment-induced declines in total sugar, their effects were negated, likely due to disrupted metabolic pathways (Godlewska and Ciepiela, 2018; Tlahig et al., 2020). The reduction in sugar accumulation during the 2023 season may be attributed to elevated stress levels, as increased sugar content is often considered an indicator of stress conditions that help maintain cellular hydraulic pressure and protect macromolecules and membranes from damage (Tlahig et al., 2020).

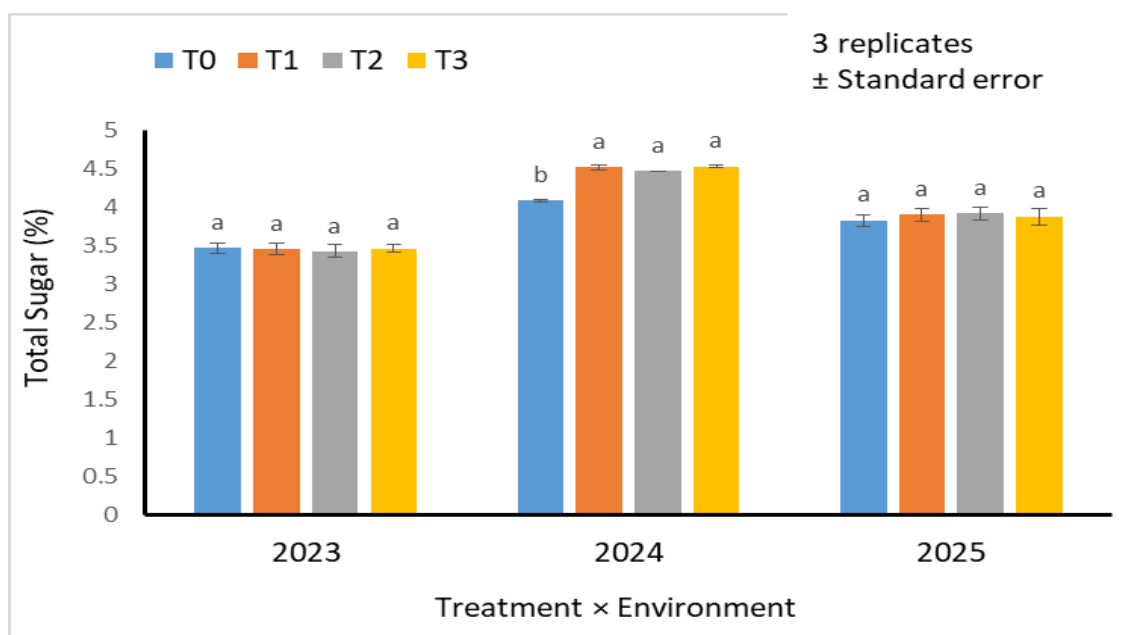


Figure 15: Interactive effect of biostimulant treatment x environment (B x E) on total sugar of alfalfa crop cultivated over a three-year period (Debrecen, 2023–2025).

Note: Columns represent annual mean values of genotype (2) and repetitions (3) $\pm$ standard error ($n = 6$). Different letters mean significant differences between treatments at $p = 5\%$

4.3.5 Differential effects of biostimulant treatments and environmental conditions on total carbohydrates in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023-2025)

Total carbohydrate results follow similar trends observed in the previous quality parameters. The statistical analysis of variance shows no significant ($p > 0.05$) effect of the treatments and genotype variation on the total carbohydrate during the 2023 and 2025 cropping seasons. Whereas a significant ($p < 0.001$) effect of these factors was noted during the 2024 cropping season. In 2024, even though significant differences existed, the application of the treatments exhibited a decline in total carbohydrate, specifically, a decrease of 12.5%, 10% and 8.5% was observed under T3, T2 and T1 compared to the control (T0) in the Magor genotype (**Figure 16**). A similar decline was detected in the Hunor genotype, with total carbohydrate decreasing from 8.2% at T1 to 10.1% at T3 when compared to the control (**Figure 16**). Meanwhile, in the 2023 cropping season, even though no significant difference existed, total carbohydrate in Hunor was slightly improved, ranging from 1.2% at T2 to 2.1% at T3 compared to the control (T0). However, a decline in total carbohydrate was observed in Magor following the application of biostimulants, ranging from -0.32% at T1 to -1.8% at T1 (**Table 15**). In addition, the 2025 cropping season followed a similar trend as observed in the 2024 cropping season.

Total carbohydrate in Magor decreased from 1.8% at T1 to 3.5% at T2, whereas in Hunor, we also noted a decrease in total carbohydrate ranging from 1.1% at T1 to 2.6% at T3, compared to the control (T0) (**Table 15**). Overall, the studies show that the application of biostimulant treatment resulted in a decline in total carbohydrate under mild and moderate drought stress conditions. Even though a significant difference existed, particularly under the 2024 cropping season, amongst treatment and genotype, which shows the genotypes responded differently to biostimulant treatments, however, the effectiveness of these treatments did not translate into enhancement of the total carbohydrate of the alfalfa plant. The decrease in total carbohydrate observed in the alfalfa treated with biostimulant (T1, T2 and T3) under these drought conditions may be partly explained by enhanced vegetative growth stimulated by these biostimulant treatments. Evidence from previous studies have shown that the application of microbial or natural plant biostimulants promotes cell division, leaf expansion and root proliferation even under suboptimal water availability (Calvo et al., 2014; Du Jardin, 2015; Samuels et al., 2022). Rouphael and Colla (2020) reported that microbial and seaweed extract-based biostimulants often accelerate the growth process, increasing the metabolic demand for carbon substrates. Similarly, Bulgari et al. (2019) highlighted that plants treated with biostimulants allocate more assimilate toward structural biomass than the storage pool. These findings support the reduced carbohydrate concentration observed in these alfalfa genotypes, reflecting increased utilisation for growth rather than impaired photosynthetic performance.

Table 15: Effect of biostimulant treatment x genotype on total carbohydrates of alfalfa crop cultivated during the 2023 and 2025 cropping seasons (Debrecen, 2023 and 2025)

Treatments	2023			2025		
	Magor	Hunor	Mean	Magor	Hunor	Mean
T0	37.49 ±0.34	36.06 ±0.66	36.77 ±0.50	35.27 ±0.57	35.00 ±0.80	35.13 ±0.69
T1	37.37 ±0.72	36.49 ±1.09	36.93 ±0.91	34.60 ±1.01	34.60 ±1.05	34.60 ±1.03
T2	37.04 ±0.62	36.51 ±1.23	36.77 ±0.92	34.03 ±1.08	34.33 ±0.84	34.18 ±0.96
T3	36.81 ±0.69	36.83 ±0.23	36.82 ±0.46	34.40 ±0.98	34.10 ±1.05	34.25 ±1.02
Means	37.17 ±0.59	36.47 ±0.80		34.57 ±0.91	34.50 ±0.93	

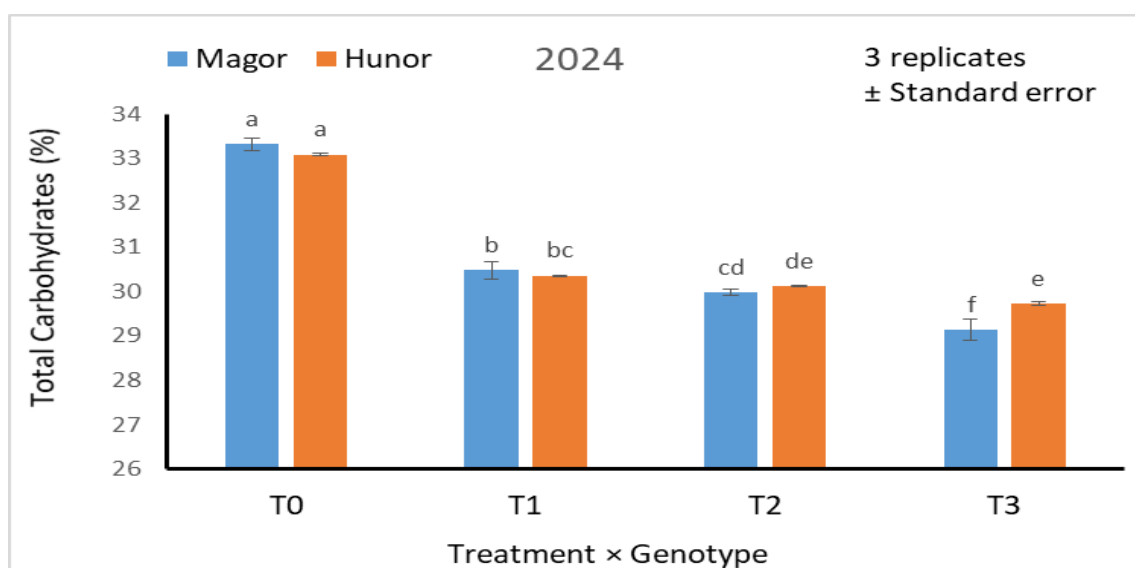


Figure 16: Interactive effect of biostimulant treatment x genotype (B x G) on total carbohydrates of alfalfa crop cultivated during the 2024 cropping season (Debrecen, 2024).

Note: Columns represent annual mean values of biostimulant treatment (4) and repetitions (3) $\pm$ standard error ($n = 12$). Different letters mean significant differences between treatments at $p = 5\%$

4.3.6 Interactive effects of varietal divergence and environmental condition on total carbohydrates of alfalfa across three cropping seasons (2023 - 2025)

In terms of complex evaluations, two-way and three-way analyses reveal only a significant ($p > 0.05$) effect existed for biostimulant treatment x environment (two-way interaction), indicating that the performance of these treatments varied across experimental years (**Figure 17**). In 2023, total carbohydrate slightly increased with biostimulant treatments, with T1 and T3 showing the highest values of 0.4% and 0.1% compared to the control (T0). In 2024 and 2025, the response of these treatments was less pronounced compared to the 2023 cropping season. In 2024, total carbohydrates were much lower than in 2024 and 2025; in fact, total carbohydrates declined from -8.3% under T1 to -11% under T3 in the 2024 cropping season, whereas a reduction from -1.5% under T1 to -2.7% under T2 was observed in the 2025 cropping season, which highlights the negative effects of these treatments on alfalfa total carbohydrates. Generally, the effectiveness of these treatments was pronounced, particularly in the 2023 season, with marginal improvement in total carbohydrates in the first-year experiment compared to the later seasons. Comparatively, 2023 was classified as a mild drought stress condition and saw an enhancement in total carbohydrate by 19.6% and 6.6% compared to the 2024 and 2025 cropping seasons, suggesting that the application of these treatments (T1, T2, and

T3) under mild drought stress enhances the alfalfa's ability to allocate carbon towards defensive structures and root elongation, ensuring that total carbohydrate reserves are maintained or increase even when shoot protein or sugar content drops. These findings are consistent with previous evidence demonstrating that biostimulants are most effective under mild to moderate stress conditions (Calvo et al., 2014; Bulgari et al., 2019). The 2023 cropping season was characterized by mild drought; we observed a slightly increased total carbohydrate content following the application of biostimulant treatment. These findings align with studies by Drobek et al. (2019) and Calvo et al. (2014), who found that biostimulants enhance carbon assimilation and osmotic adjustment when plants retain sufficient physiological activity. However, under the more intense drought conditions of the 2024 and 2025 cropping seasons, the effectiveness of these biostimulant treatments reduced total carbohydrates in alfalfa, supporting the earlier studies by Nardi et al. (2016) and Van Oosten et al. (2017), who observed that severe water limitation restricts photosynthesis and carbohydrate metabolism, thereby limiting biostimulant efficacy. Moreover, the seasonal variation likely contributed to these differences, as highlighted by Yakhin et al. (2017) and Bulgari et al. (2019), who noted that environmental factors strongly influence biostimulant performance in field conditions. Overall, the results contribute to the broader understanding that carbohydrate accumulation is a critical drought tolerance mechanism and that biostimulant application can support this process primarily under mild drought stress in alfalfa cultivation.

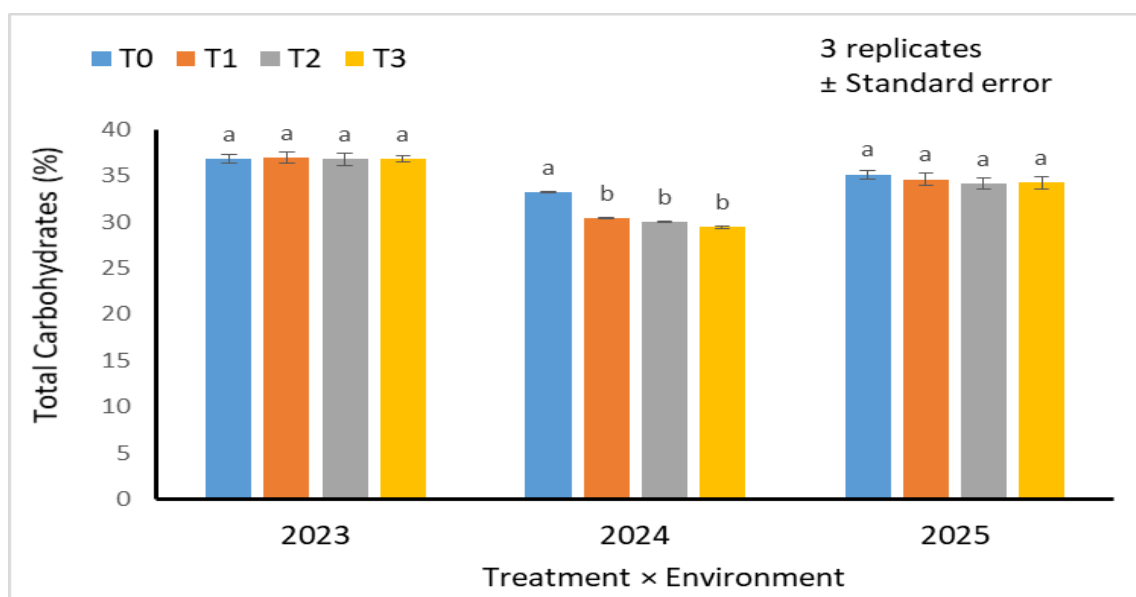


Figure 17: Interactive effect of biostimulant treatment x environment (B x E) on total carbohydrate of alfalfa crop cultivated over a three-year period (Debrecen, 2023 – 2025).

Note: Columns represent annual mean values of biostimulant treatment (4) and repetitions (3) $\pm$ standard error ($n = 12$). Different letters mean significant differences between treatments at $p = 5\%$

4.3.7 Differential effects of biostimulant treatments and environmental conditions on Fibre content in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023-2025)

Data regarding fibre content also follow similar trends observed in the previous quality parameters under three cropping seasons. The statistical analysis of variance shows no significant ($p > 0.05$) effect of the treatments and genotype variation on the fibre content during the 2023 and 2025 cropping seasons. Whereas a significant ($p < 0.001$) effect of these factors was noted during the 2024 cropping season. In 2024, the application of the treatments exhibited a significant increase in fibre content by 11%, 10.4%, and 3.5% observed under T2, T3, and T1, respectively, compared to the control (T0) in the Magor genotype. A similar increase was detected in the Hunor genotype, with fibre content reaching the maximum of 31.8% under T2, followed by 10.1% under T3 and 2.4% under T1 compared to the control (**Figure 18**). These increases in fibre content could be due to the ability of these treatments, particularly T2, to trigger the genotypes's natural stress tolerance mechanism, particularly suitable for adapting to water stress. Meanwhile, in the 2023 cropping season, even though no significant difference existed, fibre content in Magor was slightly improved, ranging from 2.9% at T2 to 4.3% at T3, whereas T1 decreased fibre content by 1.2% compared to the control (T0). However, a decline in fibre

content was observed in Hunor following the application of biostimulants, ranging from –0.7% at T2 to –6.3% at T3 (**Table 16**). In addition, the 2025 cropping season followed a similar trend as observed in the 2023 cropping season. Fibre content in Magor increased from 2% at T1 to 6.9% at T2, whereas in Hunor, we also noted a decrease in fibre content ranging from 2.2% at T2 to 4.2% at T1, compared to the control (T0). Overall, the study findings across the three seasons highlight the strong effects of environmental variability on the response of alfalfa fibre content to biostimulant treatment applications. The lack of significant genotype, biostimulant and interactive effects observed in the 2023 and 2025 cropping seasons suggests that the growing conditions during these seasons did not induce sufficient physiological stress to differentiate the response between genotypes or to enhance the action of biostimulants applied. Similar observations have been reported in forage crops, where biostimulants often show minimal impact on fibre fraction under favourable environmental conditions (Smith et al., 2019). However, under stable conditions, fibre deposition tends to follow genetically regulated pathways, resulting in limited treatment-related variation. In contrast, the observed significant effect in the 2024 cropping season demonstrates that biostimulants exert stronger impacts under moderate stress conditions. Several pieces of evidence from literature have shown that biostimulants enhance plant metabolic activity, stress tolerance, and cell wall modification when plants are exposed to or experience water deficit, heat stress, or nutrient imbalance (Calvo et al., 2014; Du Jardin, 2015; Rady et al., 2021). These stress conditions can increase the plants reliance on external stimulatory inputs, making the biostimulant effects more visible, as observed in T2 increasing fibre content by 11% in Magor and 31.8% in Hunor. In addition, the significant interactive effect (genotype x biostimulant) observed in 2024 cropping further supports findings from many studies demonstrating that genotypes differ in their ability to use biostimulant-derived signals, especially under stress-inducing environments (Ertani et al., 2009; Zhang et al., 2018). These suggest that the physiological responsiveness of these alfalfa genotypes is not uniform and becomes even more pronounced when environmental pressures activate stress-related metabolic pathways.

Table 16: Effect of biostimulant treatment x genotype (B x E) on fibre content of alfalfa crop cultivated during the 2023 and 2025 cropping seasons (Debrecen, 2023 and 2025)

Treat- ments	2023			2025		
	Magor	Hunor	Mean	Magor	Hunor	Mean
T0	66.50 ±3.23	63.30 ±4.20	64.90 ±3.71	70.67 ±3.24	73.43 ±0.58	72.05 ±1.91
T1	65.70 ±2.57	62.10 ±5.98	63.90 ±4.28	72.10 ±3.48	70.33 ±1.15	71.21 ±2.31
T2	68.40 ±3.20	62.80 ±4.38	65.60 ±3.79	75.57 ±4.84	71.80 ±0.20	71.65 ±2.52
T3	69.40 ±2.64	59.30 ±2.40	64.35 ±2.52	72.57 ±0.49	71.17 ±2.11	73.37 ±1.30
Means	67.50 ±2.91	61.87 ±4.24		72.46 ±3.01	71.68 ±1.01	

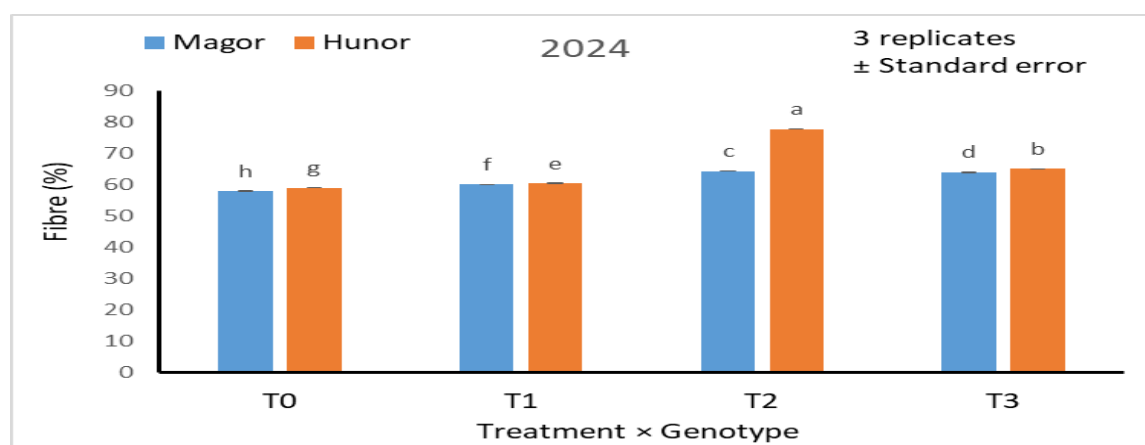


Figure 18: Interactive effect of biostimulant treatment x genotype (B x E) on fibre content of alfalfa crop cultivated during the 2024 cropping season (Debrecen, 2024).

Note: Columns represent annual mean values of biostimulant treatment (4) and repetitions (3) ± standard error (n = 12). Different letters mean significant differences between treatments at p = 5%

4.3.8 Interactive effects of biostimulant treatment and environmental condition on fibre content of alfalfa across three cropping seasons (2023-2025)

Based on the combined evaluation, our analysis reveals a significant ($p < 0.05$) effect existed for biostimulant treatment x environment (two-way interaction) and genotype variation x environment; however, no significant effect was detected for biostimulant x genotype variation or biostimulant x genotype variation x environment (B x G x E) three-way interactions. The (**Figure 19**) below illustrates the summary of the biostimulant treatment x environment observed for fibre content in alfalfa, indicating that the effectiveness of these treatments varied across the three experimental seasons. In 2023, we observed a decline in fibre content following the application of biostimulant treatments compared to the control. This reduction ranged from 0.8% at T3, 1.1% at T2

and 1.5% at T1, respectively. In the 2024 season, a significant increase in fibre content was observed following the application of biostimulant treatments, with T2 significantly increased fibre content by 21.5%, followed by T3 with 10.3%, whereas a 3% increase was also noted by T1 when compared to the control (T0). In 2025, however, the response was less pronounced than in 2024; biostimulant treatment application increased fibre content only at T3 by 1.8% compared to the control, whereas a decrease of 0.5% and 1.2% was also observed under T2 and T1, respectively. Overall, the strongest response of these treatments observed in the 2024 cropping season might be due to the ability of these treatments to boost the metabolic pathways that enhance fibre concentrations. However, the significant x environment interactions observed highlight that the effect of these treatments on fibre content of alfalfa is highly dependent on the environmental conditions. The significant increase observed in 2024 aligns with previous studies reporting that biostimulants enhance plant metabolic efficiency and cell water turnover, particularly under moderate moisture and temperature (Calvo et al., 2014; De Vasconcelos et al., 2019). In addition, under such conditions, biostimulants can stimulate enzymatic activity related to carbohydrate metabolism and cell wall remodelling, leading to modest improvements in forage quality. In contrast, the limited responses in the 2023 and 2025 cropping seasons suggest that severe abiotic stresses suppressed biostimulant efficacy, as plants shifted metabolism toward stress-avoidance pathways at the expense of growth optimisation and fibre accumulation (Rouphael and Colla, 2020; Boutahiri et al., 2024). Similarly, under adequate moisture or rainfall as likely prevailed in those years (2023 and 2025 cropping seasons), the effectiveness of biostimulants in strongly triggering fibre-enhancing pathways is limited, since plants experience minimal stress (Boutahiri et al., 2024). Thus, the environmental constraints of the 2023 and 2025 cropping seasons likely overrode any potential biostimulant-mediated improvements. These findings reinforce the notion that biostimulants can contribute to improving forage quality, but their effects are not consistent across years and can be explained with the context of seasonal climatic variability (Yakhin et al., 2017).

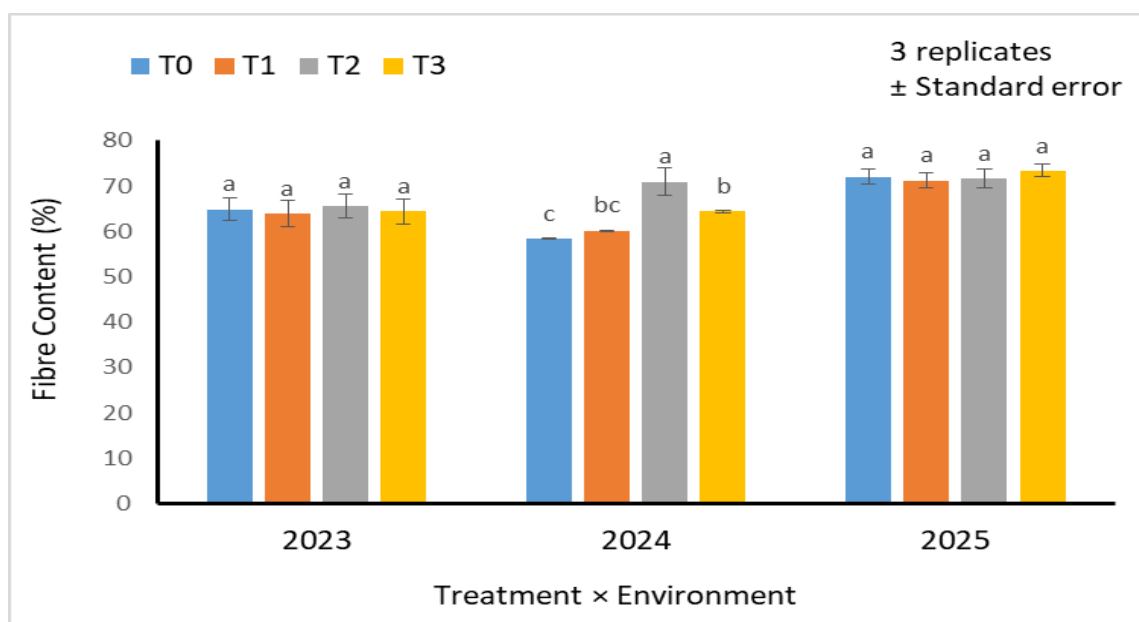


Figure 19: Interactive effect of biostimulant treatment x environment (B x E) on fibre content of alfalfa crop cultivated over a three-year period (Debrecen, 2023 – 2025). *Note: Columns represent annual mean values of biostimulant treatment (4) and repetitions (3) ± standard error (n = 12). Different letters mean significant differences between treatments at p = 5%*

4.3.9 Interactive effects of genotypic divergence and environmental conditions on fibre content alfalfa across three cropping seasons (2023-2025)

Furthermore, a significant genotype x environment variation was observed in fibre contents. Genotypes significantly differed in fibre content response across the three cropping seasons, indicating that environmental conditions affected the fibre concentration in these alfalfa genotypes. In 2023 cropping season, Magor demonstrated a significant increase in fibre content by 9% over Hunor. Similarly, in 2025 Magor maintained a consistently higher fibre content of 1.1% over Hunor, exhibiting a stable performance across these two cropping seasons. However, during 2024 cropping season, Hunor significantly outperformed Magor by increasing fibre content by 6.5% (**Figure 20**). Overall, the pronounced genotype x environment interaction observed for fibre content in alfalfa, evidenced by Magor outperforming Hunor by 9% in 2023 and 1.1% in 2025, yet fall behind by 6.5% in 2024, highlights substantial genetic variability in fibre plasticity under fluctuating environmental conditions. This G x E interaction has been extensively documented in alfalfa and other perennial forages, where genotype-specific responses in forage quality traits, such as lignin deposition under drought stress, are profoundly shaped by climatic variability, often compromising digestibility and nutritional value (Lamb et al., 2014; Annicchiarico et al., 2015a). Furthermore, Hunor,

which was noted with an increase in fibre content during the 2024 growing season (moderate drought stress), likely possesses more robust physiological mechanisms for regulating cell wall biosynthesis under this condition. Conversely, Magor showing a high improvement in fibre under mild droughts while decreasing fibre under moderate stress suggests that it may be more sensitive to drought-induced delignification. Numerous studies have shown that environmental stress conditions, especially water deficit, lead to increased lignin accumulation in alfalfa stems, which decreases digestibility and overall forage quality (Hoveland and Monson, 1980; Xu et al., 2023). In addition, the pronounced increase in fibre content in Hunor genotype during the 2024 cropping season (moderate drought) is consistent with other studies emphasizing that the tendency of alfalfa to increase lignin content under stress is a response linked to enhanced structural reinforcement and oxidative stress protection (Basu et al., 2016). This underscores the importance of selecting genotypes with stable fibre profiles across environments, especially in regions prone to climatic variability. Overall, the interaction results suggest that combining environmentally stable genotypes with biostimulant strategies may improve forage quality, but the success of such approaches will depend heavily on seasonal cropping conditions.

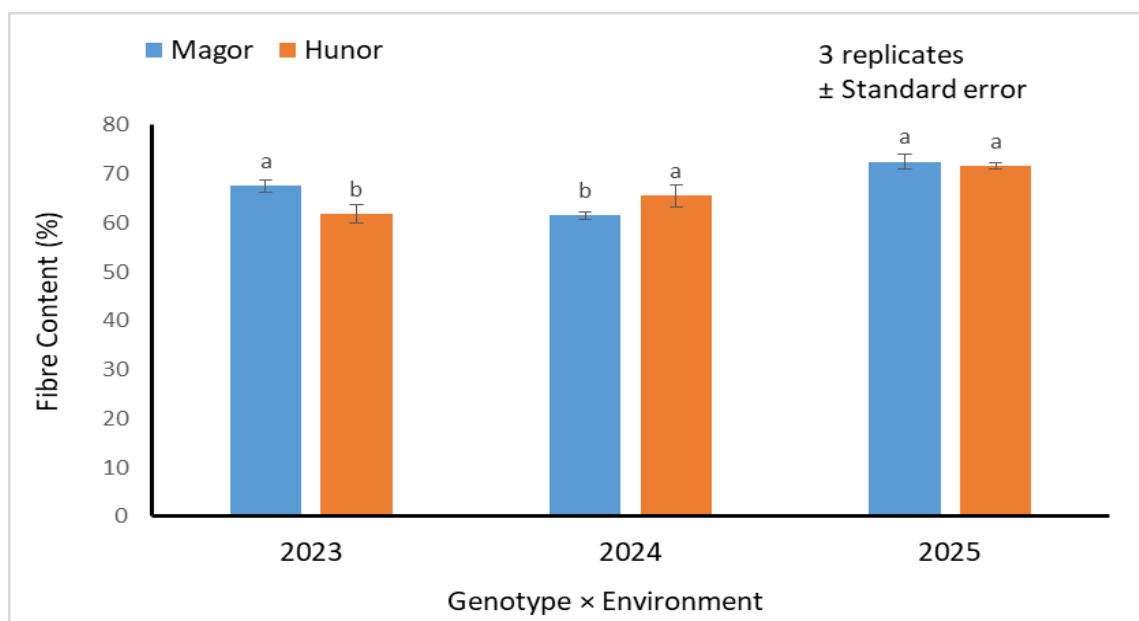


Figure 20: Interactive effect of genotype x environment (G x E) on fibre content of alfalfa crop cultivated over a three-year period (Debrecen, 2023–2025).

Note: Columns represent annual mean values of biostimulant treatment (4) and repetitions (3) ± standard error (n = 12). Different letters mean significant differences between treatments at p = 5%

4.4 Correlation analysis of quality traits and total dry matter yield of alfalfa cultivated across three cropping seasons (2023-2025).

Correlation analysis revealed significant relationships between quality parameters and total dry matter yield across three cropping seasons. These patterns indicate how biostimulants modulate alfalfa physiological responses to drought. **Table 17** shows how biostimulant application under contrasting drought conditions affected biochemical composition and total dry matter (TDMY). Correlation coefficients indicated that protein content had the strongest positive correlation with TDMY ($r = 0.449$, $p = 0.02$), likely due to biostimulants enhancing nitrogen assimilation, enzyme activity, and metabolic efficiency. This supports higher protein synthesis, photosynthesis, and growth under water deficit. Such enhancements are pivotal for bolstering overall plant productivity and resilience, particularly given that biostimulants improve nutrient uptake and chlorophyll content, which are crucial for photosynthetic efficiency (Appiah et al., 2024a). The correlation coefficients showed that fibre content exhibited a moderate positive correlation with TDMY ($r = 0.37$, $p = 0.07$), suggesting biostimulants sustained structural growth by promoting carbon allocation to cellulose and hemicellulose for cell wall development. Conversely, previous research has indicated that increased dry matter weight can also contribute to crude fibre content (He et al., 2022). This structural reinforcement helps plants maintain turgidity and mechanical strength, thereby improving resilience to environmental stressors (Malinowska and Wiśniewska-Kadżajan, 2023). However, total sugars had a weak positive correlation with TDMY ($r = 0.136$, $p = 0.52$), reflecting enhanced osmotic adjustment; sugars accumulate to stabilize membranes and maintain turgor, supporting limited growth. Conversely, the correlation between average leaf area and in vitro digestibility was moderately negative, suggesting that while increased leaf expansion boosts biomass, it may concurrently reduce nutritional quality (Temoche-Socola et al., 2025). This implies that optimizing for biomass alone may not always align with maximizing forage quality, particularly digestibility, where a balance between growth and nutrient partitioning is crucial. In contrast, total carbohydrates exhibited a negative correlation with TDMY ($r = -0.518$). High carbohydrate levels indicate reduced growth and inefficient utilization for structural or metabolic processes, marking metabolic stagnation under drought. This inverse relationship underscores a potential trade-off, where increased carbohydrate storage under stress may reflect redirection of resources from biomass accumulation toward survival mechanisms, ultimately limiting yield.

Interrelationships among quality parameters also showed positive correlations, particularly between total sugar and both protein and fibre content ($r = 0.689$, $p = 0.001$) and ($r = 0.079$, $p = 0.71$). These suggest biostimulants coordinate metabolism, with sugars aiding protein synthesis and both supporting structural biomass in alfalfa cultivation. Such synergistic effects, particularly enhancement of lateral root development and associated phytohormones like auxins and cytokinins, contribute to improved nutrient acquisition and stress tolerance, bolstering overall plant vigor and productivity (Akdağ et al., 2024). In contrast, we observed negative correlations between total carbohydrates and protein ($r = 0.884$, $p = 0.001$), fibre ($r = 0.419$, $p = 0.041$), and sugars ($r = 0.525$, $p = 0.008$), reinforcing that their accumulation inhibits pathways and reduces activity. Overall, biostimulants promote metabolic resilience in alfalfa under drought. Genotypes maintaining high protein, fibre, and sugars sustained biomass, while high carbohydrates indicated slowdown. This highlights biostimulants' role in efficient carbon and nitrogen use for yield and quality.

Table 17: Correlation Matrix of Quality parameters and Total Dry Matter Yield of Alfalfa under natural drought stress condition over a three-cropping season (Debrecen, 2023-2025)

	Total sugar	Total carbo	Fibre	Protein	Total DMY
Total sugar	-				
Total carbo	-0.525	-			
Fibre	0.079	-0.419	-		
Protein	0.689	-0.884	0.360	-	
Total DMY	0.136	-0.519	0.372	0.449	-

**Total carbo = total carbohydrate; DMY = dry matter yield, significant at $p = 0.05$, significant at $p = 0.001$

4.5 Differential effects of biostimulant treatments and environmental conditions on protein yield in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023-2025)

Evaluating the protein yield response of alfalfa genotypes to biostimulant treatments across three cropping seasons under drought stress revealed a significant improvement in protein yield per harvested area. The analysis of variance showed no significant ($p = 0.343$) effect of the treatments on protein yield in 2023, classified as mild drought. However, in both the 2024 and 2025 cropping seasons, classified as moderate and medium drought, respectively, the analysis revealed highly significant ($p < 0.05$) effects

of the applied treatments on protein yield of alfalfa genotypes and the means are summarised in (**Table 18**). No interaction effect existed across three seasons. Numerically, in the 2023 cropping season, although no significant effects existed, T3 outperformed the other treatments by increasing protein yield by 12.7%, followed closely by T2 and T1 with 9.9% and 9.5% compared to the control. In the 2024 cropping season, T2 achieved the highest protein yield of 9982 kg ha⁻¹, followed by T3 and T1 with 9428 kg ha⁻¹ and 9273 kg ha⁻¹, reflecting significant increases of 41.5% for T2, 33.7% for T3, and 31.5% for T1 compared to the control. A similar trend was observed in the 2025 cropping season, where T2 maintained its outstanding performance by increasing protein yield by 42.8%, followed closely by T3 with 39.8% and T1 with 27.4% compared to the control.

Overall, our results demonstrate that biostimulant treatments significantly enhanced protein yield of alfalfa genotypes under moderate and medium drought conditions across the 2024-2025 cropping seasons, with numerically positive though non-significant effects under mild drought in 2023. The response of alfalfa genotypes to biostimulant treatments varied with drought severity, indicating that the effectiveness of these treatments is closely linked to environmental stress intensities. Under mild drought stress conditions in the 2023 cropping season, no significant treatment effect on protein yield was observed; however, a slight enhancement was noted with T3 increasing protein yield by 12.7%, followed closely by T2 and T1 with 9.9% and 9.5% compared to the control. This suggests that under mild water stress, the inherent drought tolerance and physiological resilience of alfalfa may reduce the measurable impact of external biostimulant inputs. This observation aligns with previous studies demonstrating that the effects of biostimulants often become more pronounced under moderate or severe abiotic stress conditions where plant physiological limitations are stronger (Yakhin et al., 2017; Sario et al., 2025). In contrast, the 2024 and 2025 cropping seasons, classified as moderate and medium drought conditions, showed a highly significant impact of treatments on protein yield of alfalfa, with T2, T3, and T1 significantly increasing protein yield by 41.5%, 33.7%, and 31.5% in 2024, and by 42.8%, 39.8%, and 27.4% in 2025 relative to the control, respectively. This substantial improvement indicates that biostimulant application substantially enhanced alfalfa productivity under drought conditions, likely through enhanced physiological and metabolic processes that support plant growth and protein synthesis. Several studies have reported that biostimulants improve nutrient uptake efficiency, stimulate root development, and enhance plant stress tolerance through

the activation of metabolic pathways and antioxidant defense systems (Calvo et al., 2014; Yakhin et al., 2017; Roupael and Colla, 2021; Akdağ et al., 2024). According to Roupael and Colla, (2021) biostimulants can enhance plant resilience to abiotic stress by regulating hormonal balance, improving water use efficiency, and promoting root architecture development. In drought-prone environments, these mechanisms such as increased root growth, better nutrient uptake, and stress tolerance (Calvo et al., 2014; Du Jardin, 2015) allow crops to maintain metabolic activity and biomass accumulation, ultimately supporting higher protein yield. In the case of alfalfa, a protein-rich forage crop, improved nitrogen assimilation and sustained leaf growth under stress conditions likely contributed to the observed increase in protein yield.

Furthermore, the superior performance of treatment T2, increasing protein yield by 41.5% to 42.8% across the moderate and medium drought seasons, suggests that the composition or concentration of this treatment optimized plant physiological responses to water deficit. Previous studies have demonstrated that certain microbial or non-microbial biostimulants can significantly enhance forage crop productivity under drought by improving osmotic adjustment, photosynthetic efficiency, and nitrogen metabolism (Akdağ et al., 2024). These mechanisms are particularly relevant for alfalfa because its protein yield is strongly dependent on nitrogen fixation and overall biomass production. The progressive increase in treatment effectiveness from mild to moderate drought conditions observed in this study is consistent with earlier findings indicating that biostimulants often express their greatest benefits under stress conditions rather than under optimal environments (Roupael and Colla, 2021; Akdağ et al., 2024). Overall, our results demonstrate that biostimulant treatments can play a significant role in improving protein yield in alfalfa production systems under water deficit conditions. The consistent superiority of treatment T2 across multiple cropping seasons suggests that T2 may provide a practical strategy for enhancing forage productivity and resilience in drought-affected agricultural systems. These findings contribute to the growing body of evidence supporting the integration of biostimulants into sustainable crop management practices, particularly in regions where climate variability and water scarcity threaten forage production.

Table 18: Effect of biostimulant treatments on protein yield (kg ha⁻¹) of alfalfa under natural drought stress condition over a three-cropping season (Debrecen, 2023-2025)

TREATMENT	2023	2024	2025
T0	5498 ± 324.8	7051 ± 177.1	5194 ± 270.2
T1	6021 ± 277.5	9273 ± 114.9	6620 ± 362.7
T2	6046 ± 237.1	9982 ± 228.6	7420 ± 533.7
T3	6198 ± 287.4	9428 ± 178.3	7262 ± 585.1
LSD at (5%)	842.4	455.2	853.6

4.6 Differential effects of genotypic variation and environmental conditions on protein yield in *Medicago sativa* L. and *Medicago x varia* Martyn across three cropping seasons (2023-2025)

Evaluation of the effect of genotypic variation on protein yield across three cropping seasons under drought stress revealed significant improvements in protein yield per harvested area overall. Analysis of variance showed no significant genotypic effect on protein yield in the 2023 cropping season (mild drought). However, in the 2024 (moderate drought) and 2025 (medium drought) seasons, genotypic variation exerted highly significant effects on alfalfa protein yield, with means summarized in **Table 19**. Although non-significant in 2023, Magor numerically outperformed Hunor by increasing protein yield 9.8%. In 2024, Magor achieved the highest protein yield of 9112 kg ha⁻¹, compared to Hunor's 8755 kg ha⁻¹, reflecting a 4.1% increase. A similar trend persisted in 2025, with Magor boosting protein yield by 26.4% over Hunor. Overall, our results demonstrated that genotypic variation significantly enhanced the protein yield of alfalfa genotypes under varying drought intensities across the 2023 - 2025 cropping seasons. Protein yield is a key indicator of forage quality and productivity in alfalfa, particularly under water-limited environments where drought stress can strongly influence biomass production and nitrogen metabolism. Previous evidence has shown that water deficit can significantly reduce biomass accumulation, alter nitrogen metabolism, and consequently decrease crude protein content in alfalfa forage (Erica et al., 2009; Kaciene et al., 2022). However, our current study revealed no significant effect of genotypic variation on protein yield under mild drought conditions during the 2023 cropping season. This finding suggests that under favorable moisture conditions, different alfalfa genotypes may exhibit similar physiological performance in terms of biomass production and protein accumulation. This finding aligns with previous studies demonstrating that the effects of genotype on forage quality traits are often less pronounced under non-severe stress conditions because plants can maintain normal metabolic processes when water availability is adequate (Liu et al., 2018a; Lin et al., 2020). Nevertheless, the numerical

superiority of Magor, achieving 9.8% higher protein yield over Hunor, suggests inherent genetic potential for higher productivity that becomes more evident under environmental stress.

In contrast, the effectiveness of genotypic variation became highly significant under moderate drought in 2024 and medium drought in 2025 (**Table 19**). In 2024, Magor achieved the highest protein yield at 9112 kg ha⁻¹, surpassing Hunor (8755 kg ha⁻¹) by 4.1%, while in 2025, Magor increased protein yield by 26.4% over Hunor. These findings align with previous studies demonstrating that drought-tolerant alfalfa cultivars consistently produce higher crude protein levels and biomass than drought-sensitive ones under drought stress, due to genetic variation in physiological traits such as water-use efficiency, root architecture, osmotic adjustment, and nitrogen fixation capacity (Liu et al., 2018a; Nadeem et al., 2019). For instance, drought stress reduces crude protein fractions in alfalfa through combined limitations in water availability and N fixation, with tolerant genotypes maintaining higher protein via better N uptake and fixation (Liu et al., 2018b; Kaciene et al., 2022). The increasing advantage of Magor under more severe drought conditions, particularly the 26.4% higher protein yield in 2025, indicates a strong genotype x environment interaction. Drought-tolerant alfalfa genotypes activate physiological and biochemical mechanisms such as enhanced antioxidant enzyme activity, improved photosynthetic performance, reduced oxidative damage, deeper root systems, and better regulation of carbon/nitrogen metabolism to sustain biomass and protein accumulation under stress (Erice et al., 2009; Nadeem et al., 2019; Chen et al., 2024), genetic control of these traits is supported by genomic studies identifying molecular markers associated with alfalfa biomass yield and forage quality under drought, confirming that drought tolerance and productivity are complex, polygenic traits (Lin et al., 2020). Overall, these results underscore that genotypic variation critically determines alfalfa protein yield under drought stress, with differences minimal under mild drought but increasingly pronounced as severity escalates. This pattern emphasizes selecting drought-resilient genotypes like Magor, which consistently outperformed Hunor across seasons, to ensure stable forage production and high protein yield in water-scarce regions.

Table 19: Effect of genotypic variation on protein yield (kg ha⁻¹) of alfalfa under natural drought stress condition over a three-cropping season (Debrecen, 2023-2025)

Genotype	2023	2024	2025
Magor	6220 ± 164.5	9112 ± 358.9	7398 ± 414.4
Hunor	5661 ± 210.9	8755 ± 349.3	5849 ± 207
Lsd (5%)	595.7	321.9	603.6

4.7 Complex evaluation of biostimulant treatment, genotype, year and their interactions on protein yield alfalfa under contrasting drought condition (2023-2025)

A highly significant effects of treatment, genotype, year, treatment x year, and genotype x year interactions were observed, indicating substantial impacts on protein yield from season (partial $\eta^2 = 0.86$), biostimulant treatment (partial $\eta^2 = 0.69$), and genotype (partial $\eta^2 = 0.40$) (**Table 20**). The treatment x genotype interaction had a moderate effect size (partial $\eta^2 = 0.05$), while the treatment x year and genotype x year interactions were large (partial $\eta^2 = 0.36$ and 0.21). The three-way interaction (treatment x genotype x year) exhibited a moderate effect size (partial $\eta^2 = 0.07$), though it was not statistically significant. These results demonstrate that season, biostimulant treatments, and genotypic variation accounted for the greatest variation in alfalfa protein yield; however, genotype-specific responses to treatments were strongly modified by crop year variation, underscoring the vital role of environmental influences. This highlights the necessity of considering genotype-by-environment interactions when optimizing alfalfa cultivation practices, as differing environmental conditions can significantly alter the efficacy of specific biostimulant treatments and genotypic performance (El-Hifny et al., 2019). Other studies also demonstrated while certain biostimulants may enhance yields, their effectiveness depends on both the specific alfalfa genotype and prevailing environmental conditions, particularly annual precipitation (Godlewska and Ciepiela, 2018). This aligns with previous research emphasizing the influence of environmental factors, such as rainfall differences, on plant performance and the differential effectiveness of treatments and genotypes (Yousefi et al., 2025).

Table 20: Biostimulant treatment, genotype variation and crop year interaction effect on protein yield of alfalfa under drought stress condition (Debrecen, 2023-2025)

Source of variation	Df	F	p	Partial η^2
Treatments (B)	3	34.73	<.001	0.69
Genotype (G)	1	31.64	<.001	0.40
Season (Y)	2	153.67	<.001	0.86
B x G	3	0.95	0.423	0.05
B x Y	6	4.42	0.001	0.36
G x Y	2	6.36	0.004	0.21
B x G x Y	6	0.64	0.698	0.07

Note: df = degree of freedom, F = F-value, P = significance, partial η^2 = partial eta square

4.8 Principal component analysis of biostimulant treatments, genotype and environment (season) effects on growth, physiology, dry matter yield and quality traits in alfalfa (*Medicago sativa* L.) and variegated alfalfa (*Medicago x varia* Martyn)

4.8.1 Principal component analysis of genotype effects on alfalfa growth, physiology and dry matter yield

The Principal Component Analysis (PCA) was performed to investigate the associations between total dry matter yield, growth traits, physiological traits and biochemical traits as influenced by genotype variation, biostimulant treatments across three cropping seasons (2023-2025). The genotype variations, biostimulant treatments and variability in cropping season leads to notable shift in variables investigated. The PCA biplot illustrating the effect of genotype divergence on the growth, physiological, biochemical and total dry matter yield of alfalfa reveals a modest but observable partial separation between the two tested genotypes along the first two PCs, which explains 63.5% of the total variance, with PC1 accounting for 37.7% and PC2 accounting for 25.8% (**Figure 21**). Magor genotype was mostly distributed on the positive side of PC1, whereas Hunor genotype were more distributed towards the negative side of PC1. Although the confidence ellipses for Magor genotype and Hunor genotype display a considerable degree of overlap, indicating a partial similarity in growth, physiological, total dry matter yield and biochemical traits measured. However, Hunor genotype was more concentrated toward the negative side of the first PC and is primarily associated with higher levels of total carbohydrates and NDVI at 14 DAH. In contrast, Magor genotype tends to align positively along the first PC, showing a greater correlation with total sugar, fibre, protein, PH at both (21 and 28 DAH), NDVI at 28 DAH, NPR at 14 DAH and total dry matter

yield, suggesting that genotypes positioned on the right side of the plot were associated with higher values of these parameters. The PC2 was mainly influenced by NDVI at 14 DAH and PH at 14 DAH, which loaded positively, while NPR at 14 DAH and NPR at 28 DAH showed negative associations with this component. This suggests that PC2 differentiates genotypes based on early growth and physiological responses. The overall spatial distribution indicated that while divergences in genotypes are not as pronounced as those observed in biostimulant application effects, Magor genotypes tended to be more associated with higher yield-related and structural traits, while Hunor genotypes were more associated with carbohydrate accumulation and early vegetative indices, highlighting genotype-specific trait patterns. These patterns reflect fundamental growth, physiological, yield and biochemical variation that may be important for genotype selection, particularly in breeding programs targeting enhanced yield and nutritional profiles of alfalfa.

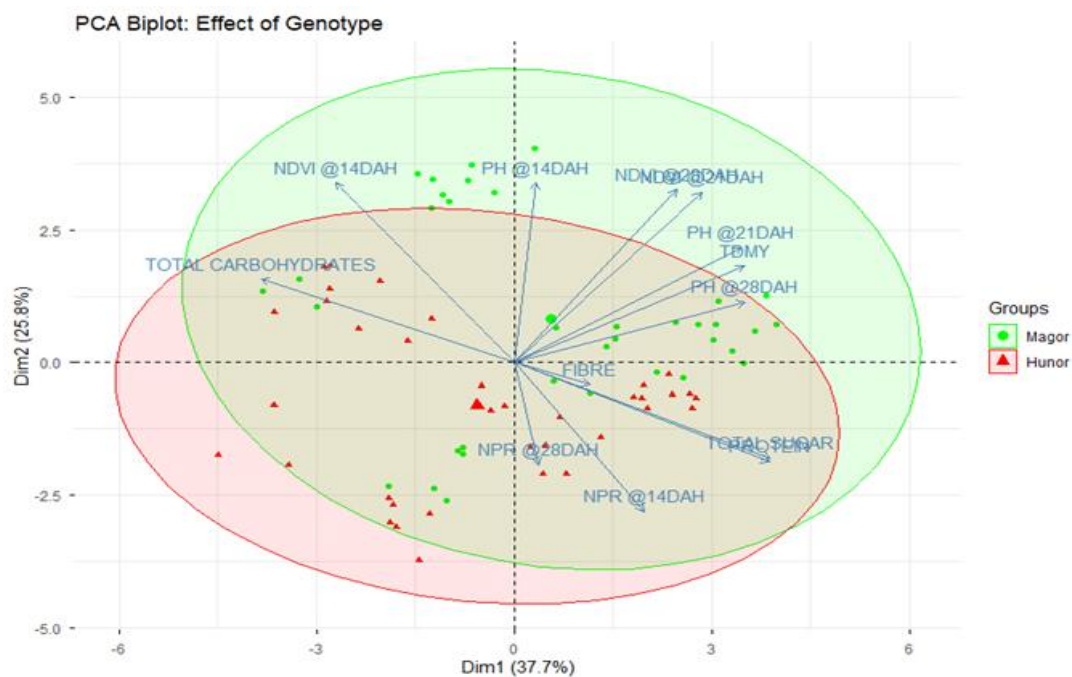


Figure 21: The principal component analysis biplot illustrating the effect of genotype divergence on the growth, physiological traits, biochemical and total dry matter yield of alfalfa. The first principal component explains 37.7% of the variance, whereas the second principal component contributes 25.8%, making 63.5% of the total variability. (Debrecen, 2023-2025).

4.8.2 Principal component analysis of biostimulant effects on alfalfa growth, physiology and dry matter yield

The results from the PCA biplot indicate that the application of biostimulants significantly affected the growth, physiological traits, biochemical and total dry matter yield of alfalfa (**Figure 22**). The distinct clustering of treatment groups suggests that specific formulations, particularly T2 and T3, substantially alter the plant's growth and biochemical profile, resulting to higher yields. The first two principal components (PC1 and PC2) explain 63.5% of the total variance, with PC1 accounting for 37.7% and PC2 for 25.8%. A clear separation is observed along PC1, particularly between the control (T0) and application of the biostimulant treatment (T1, T2, T3). The control treatment aligns more strongly with traits towards the negative side of PC1 and PC2, reveals a stronger association with total carbohydrates and NDVI at 14DAH. This indicates that plants under control conditions tended to have higher carbohydrate accumulation and early vegetation index values but were less associated with traits related to biomass production and structural development. The PCA biplot revealed noticeable differences among treatments, although some overlap was observed. T2 was mainly distributed on the positive side of the first PC, indicating a stronger association with traits related to productivity and structural components, such as plant height at 21 and 28 at DAH, NDVI at 28DAH, fibre, and total sugar and total dry matter yield. This suggests that T2 tended to promote higher biomass production and related traits.

In contrast, the application of T3 showed a distribution similar to T2 but with a slightly wider spread, indicating moderate variability among traits under this treatment. T3 was also associated with several productivity-related traits but to a lesser extent than T2. In addition, T1 was located mostly near the central region of the plot with partial overlap with T2 and T3, suggesting intermediate responses for most measured variables. The direction and magnitude of the variable vectors highlight strong positive correlations between Plant height at all stages (14, 21 and 28DAH), NDVI at 28DAH, and TDMY. In contrast, total carbohydrates and NDVI at 14 DAH exhibit an inverse relationship with these traits. The limited overlap between treatment group ellipses further supports the presence of meaningful and measurable parameters shifts. These results universally suggest that biostimulant applications, particularly T3, and T2 significantly influence the total dry matter yield and growth-related traits of Alfalfa, whereas T0 was associated

with early physiological traits and carbohydrate accumulation, highlighting clear treatment-related patterns in plant responses.

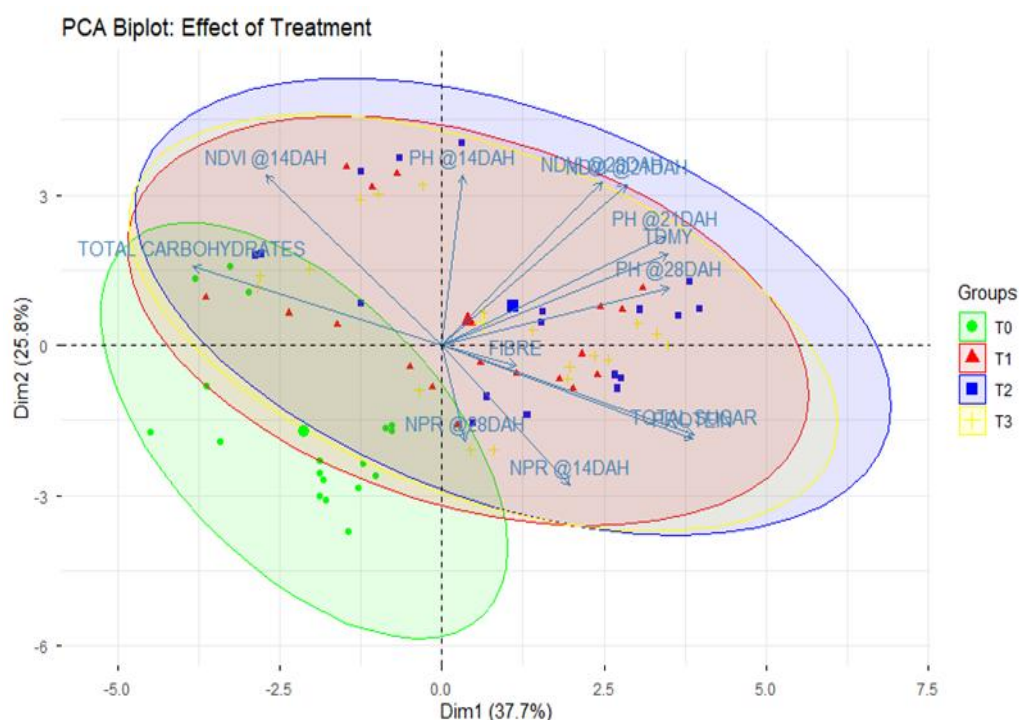


Figure 22: The principal component analysis biplot illustrating the effect of biostimulant treatments on the growth, physiological traits, biochemical and total dry matter yield of alfalfa. The first principal component explain 37.7% of variance whereas the second principal component contributes 25.8% making 63.5% of the total variability. (Debreceen, 2023-2025).

4.8.3 Principal component analysis of biostimulant effects on alfalfa growth, physiology and dry matter yield

The PCA results clearly demonstrate that seasonal variation plays a significant role in shaping the plant performance, yield and quality trait of alfalfa. Similar to the previous analysis, the first two PCAs together explain 63.5% of the total variance, with PC1 accounting for 37.7% and PC2 for 25.8% (**Figure 23**). The marked separation between the 2023, 2024 and 2025 cropping seasons along the first principal component reflects substantial differences in growth, physiological traits, biochemical and total dry matter yield of alfalfa. Samples from the 2023 season are predominantly clustered on the negative side of PC1, suggesting an association with higher levels of total carbohydrates and NDVI at 14 DAH. This indicates that plants grown during 2023 were more strongly related to early vegetative growth indicators and carbohydrate accumulation. In contrast, samples from the 2024 season are distinctly separated on the positive side of PC1 and are characterized by elevated levels of key parameters, including plant height at 21 and 28

DAH, total dry matter yield (TDMY), NDVI at 28 DAH, fibre, and total sugar. These variables are oriented toward the same quadrant as the 2024 samples, indicating improved growth performance and yield-related traits during this season.

The 2025 cropping season occupied an intermediate position between the 2023 and 2024 clusters but was slightly closer to the positive side of PC1. This suggests that plant responses in 2025 cropping season shared similarities with the productive traits observed in 2024 while still showing some overlap with characteristics associated with earlier vegetative traits.

The orientation of trait vectors further illustrated relationships among variables, such as Plant height at 14, 21, and 28 DAH, NDVI at 28 DAH, and TDMY, which were closely aligned, indicating strong positive correlations among these growth and yield-related traits. Similarly, total sugar and fibre showed a positive association. Conversely, NDVI at 14DAH and total carbohydrates were directed toward the opposite side of the plot, suggesting a negative relationship with yield-related parameters.

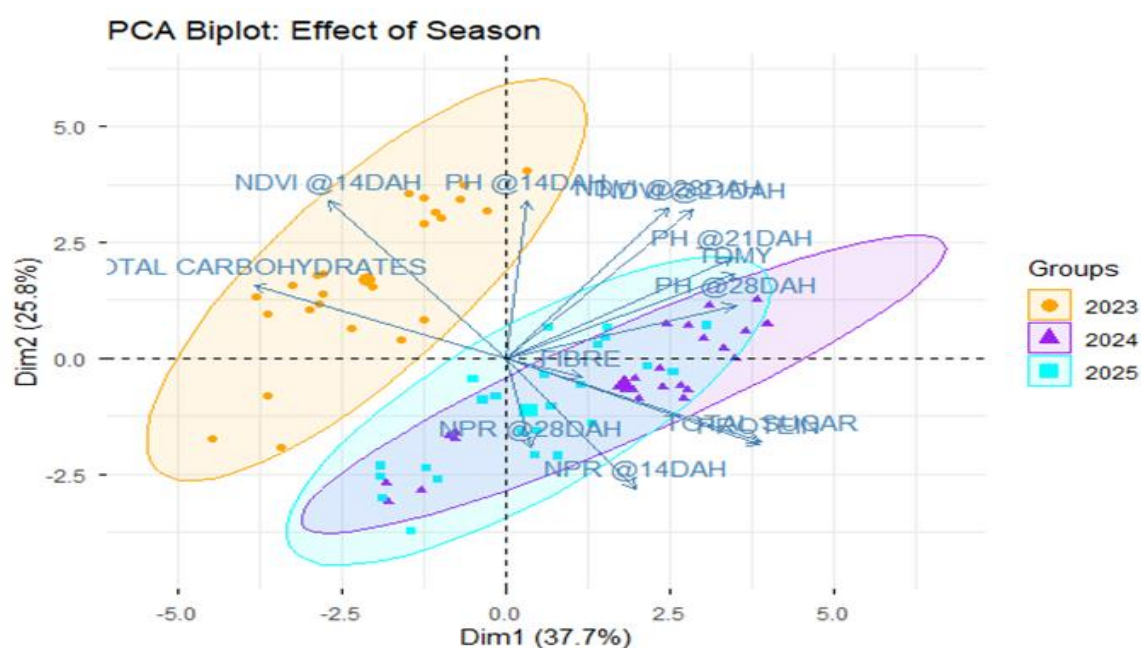


Figure 23: The principal component analysis biplot illustrating the effect of seasonal variability on the growth, physiological traits, biochemical and total dry matter yield of alfalfa. The first principal component explains 37.7% of the variance, whereas the second principal component contributes 25.8%, making 63.5% of the total variability. (Debreceen, 2023-2025).

Overall, the PCA results highlight that seasonal variation significantly influenced the distribution of traits, with 2024 showing stronger associations with growth and yield

attributes, 2023 being linked to early vegetative indices and carbohydrate accumulation, and 2025 exhibiting intermediate responses. These findings suggest that environmental conditions across seasons played an important role in shaping plant physiological and productivity traits.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

Across three cropping seasons under mild, moderate, and medium drought stress, our three-year-study demonstrated that biostimulant application, genotype variability and their interaction (genotype x biostimulant) under seasonal drought intensity exerted significant and interconnected effects on the growth, canopy vigor, physiological performance, dry matter yield, forage quality, and protein yield of *Medicago sativa* L. and *Medicago x varia* Martyn. The interaction effect of genotype x biostimulant played a vital role in shaping alfalfa response to drought stress, with important implications for productivity and forage quality. Specifically, Magor + T2 (Tricho Immun + Ino Green) was observed with the most improved net photosynthetic rates and dry matter yield across the contrasting drought stress. However, the main biostimulant treatment effects, particularly T2 consistently enhanced plant height, NDVI, and net photosynthetic rate, with effects becoming most pronounced at 21 - 28 DAH. These improvements translated into higher dry matter accumulation, improved canopy persistence, and delayed senescence, which collectively contributed to greater dry matter yield across seasons. The enhanced photosynthetic efficiency observed under these treatments (T1, T2 and T3) supports the conclusion that biostimulants strengthened carbon assimilation and metabolic resilience, enabling plants to maintain biomass production even under water-limited conditions.

Across treatments, genotypic variation played a central role in determining productivity outcomes. The Magor genotype consistently outperformed Hunor not only in growth and NDVI but also in dry matter yield, crude protein concentration, and protein yield, particularly under moderate and medium drought. Magor's superior canopy maintenance, higher chlorophyll retention, and stronger photosynthetic stability allowed it to sustain forage quality parameters such as protein content, fibre composition, and total sugar more effectively than Hunor. These results confirm Magor's greater adaptive plasticity and its ability to maintain both quantity and quality of forage under drought stress.

Seasonal drought intensity emerged as the dominant environmental factor shaping yield and quality responses. Moderate drought conditions amplified biostimulant efficacy and genotypic differentiation, while medium drought reduced overall yield potential but still allowed T2 and Magor to maintain relatively higher protein yield and forage nutritive value. Although most three-way interactions (B x G x Y) were non-significant, the few

significant cases particularly in later regrowth phases indicate that environmental variability can modulate treatment responsiveness at specific developmental stages. Overall, the integration of biostimulant application with alfalfa genotypes significantly improved biomass productivity, forage quality, and protein yield under water-limited conditions. These findings reinforce the value of biostimulants as resilience-enhancing tools and highlight the importance of genotype selection and season-specific management strategies in optimizing alfalfa productivity under increasing climatic stress.

5.2 Recommendation

- Our study advocates that farmers should prioritize these biostimulant treatments, particularly T2, to enhance the yield and nutritive value of the alfalfa crop. This is because T2 consistently improved plant height, NDVI, photosynthetic rate, dry matter yield, crude protein concentration, and protein yield across seasons. Its use is recommended for drought-prone alfalfa systems to stabilize both productivity and forage quality.
- Magor significantly outperformed Hunor in canopy vigor, biomass accumulation, forage quality traits, and protein yield. Its superior drought resilience makes it a strong candidate for breeding programs and commercial cultivation in water-limited environments.
- Farmers should consider applying biostimulant early in the regrowth stage, because the effectiveness of the treatment became most pronounced at 21–28 DAH. Early application ensures sufficient physiological activation, leading to improved biomass formation and protein synthesis.
- Moderate drought conditions amplified treatment and genotype differences, while medium drought reduced overall yield but still allowed T2 and Magor to maintain higher forage quality. Therefore, monitoring seasonal drought patterns can optimize application timing and expected outcomes.
- NDVI proved highly sensitive to canopy vigor, biomass accumulation, and quality-related traits. Therefore integrating NDVI and regular physiological monitoring can support early detection of stress and guide timely interventions.

6. NEW SCIENTIFIC RESULTS

1. The combined application of Tricho Immun + Ino Green (T2) enhanced alfalfa growth under drought stress, increasing plant height by 12.5-29.8% across different drought intensities compared with the untreated control. Genotypic differences became more pronounced with increasing drought severity, with the Magor genotype (*Medicago x varia* Martyn) exceeding Hunor (*Medicago sativa* L.) by 9.2-13.0% in plant height across different drought intensities.
2. Biostimulant treatments improved canopy performance under drought conditions. Biostimulants increased NDVI by 4-11%, with the greatest response under moderate drought (11%). The Magor genotype exceeded Hunor by 3-14%, demonstrating superior canopy maintenance under water deficit.
3. Biostimulant treatments significantly enhanced photosynthesis under drought stress, with increases of up to 86% compared with the untreated control. Tricho Immun + Ino Green (T2) increased the net photosynthetic rate by 71% under mild drought, whereas the biostimulant containing MTU[®], pidolic acid, and Si (T1) increased it by 86% under moderate drought. The greatest improvements were observed in the drought-tolerant Magor genotype.
4. Biostimulant treatments significantly increased total dry matter yield across seasons, with the greatest improvement achieved by Tricho Immun + Ino Green (T2) (+27%), followed by Tricho Immun (T3) (+23%) and T1 (+18%) compared with the untreated control. The Magor genotype produced 6-18% higher total dry matter yield and 12-26% higher protein yield than Hunor.
5. Alfalfa (*Medicago varia* Martyn and *Medicago sativa* L.) showed the strongest response to biostimulant treatments under moderate drought conditions, with NDVI increasing by over 11%, plant height by up to 18.7%, net photosynthetic rate by up to 86%, protein yield by up to 28%, and total dry matter yield by up to 22%, indicating that biostimulant treatments were most effective under moderate drought conditions.
6. A significant three-way interaction at Phase 3 (21 and 28 DAH) demonstrated that the effects of biostimulants containing MTU[®], pidolic acid, Si (T1), Tricho Immun + Ino Green (T2) and Tricho Immun (T3) on NDVI depended on both genotype and seasonal conditions.

7. Plant height and NDVI measured at 21- 28 DAH showed the strongest correlations with total dry matter yield (plant height: $r=0.553-0.639$; NDVI: $r=0.603-0.638$), indicating that these parameters are reliable predictors of alfalfa biomass production under drought conditions.
8. The negative correlation between net photosynthetic rate (NPR) and total dry matter yield ($r=-0.049- -0.203$) indicates that leaf-level photosynthesis alone is not a reliable predictor of biomass production under drought conditions. Similarly, negative correlation was found between normalized difference vegetation index and NPR ($r=-0.116- -0.755$).
9. Biostimulant treatments showed season-dependent effects on total carbohydrate content. Under mild drought, total carbohydrate content was 6.6-19.6% higher than under moderate and medium drought, indicating that biostimulant efficacy on carbohydrate accumulation was greatest under mild water deficit.
10. Total carbohydrate content was negatively correlated with total dry matter yield ($r = -0.518$), indicating that carbohydrate accumulation was associated with reduced biomass production under drought conditions.
11. Biostimulant treatments significantly improved alfalfa forage quality under drought conditions by increasing protein content by 1.4-11%, total sugars by 1.3-10.9%, and protein yield by 9.5-42%, while reducing total carbohydrate content by 1.5-11% compared with the untreated control.

7. PRACTICAL UTILISATION OF RESULTS

1. Farmers should consider using biostimulants containing MTU[®], pidolic acid, Si (T1) and Tricho Immun + Ino Green (T2) to improve alfalfa performance under drought conditions. Tricho Immun + Ino Green (T2) increased plant height by 12-37% and NDVI by 5%-11%, while both T1 and T2 enhanced the net photosynthetic rate by more than 70%, demonstrating their potential to promote growth, maintain canopy greenness, and sustain physiological activity under water deficit.
2. The lack of significant biostimulant effects at 14 DAH, followed by significant responses at 21–28 DAH across years and growth phases, indicates that biostimulants should be applied shortly after cutting rather than after the onset of visible drought symptoms, allowing sufficient time (2–3 weeks) for the activation of physiological processes.
3. For alfalfa production in drought-prone regions, Magor genotype (*Medicago x varia* Martyn) should be considered for cultivation because it consistently outperformed Hunor (*Medicago sativa* L.), maintaining 4-14% higher canopy vigor, 8-13% greater plant height, and superior photosynthetic performance and biomass yield under drought conditions.
4. Biostimulants should be applied before drought becomes severe, as their effectiveness is greatest under moderate drought conditions when plant metabolic activity is still maintained. Under moderate drought, biostimulant treatments increased NDVI by up to 11% and plant height by up to 18.7%.
5. Farmers and agronomists should use non-destructive precision agriculture tools, particularly remote sensing technologies and handheld NDVI sensors, to detect early drought stress, evaluate biostimulant effectiveness, and support fertilization decisions. These tools enable rapid assessment of alfalfa plant health and facilitate timely management interventions.
6. The biostimulants containing MTU[®], pidolic acid, Si (T1) and Tricho Immun + Ino Green (T2) can be used to maintain photosynthetic activity under drought conditions. These treatments increased the net photosynthetic rate by up to 86%, contributing to improved dry matter yield, forage quality, and protein content.
7. The combined application of the T2 treatment (Tricho Immun + Ino Green) and the Magor genotype represents a promising strategy for enhancing drought

resilience in alfalfa. Farmers and agronomists should consider this integrated approach to improve stand stability and sustain forage production under drought conditions.

8. SUMMARY

Drought stress represents the most severe abiotic constraint on alfalfa (*Medicago sativa* L. and *Medicago x varia* Martyn) productivity, persistence, and forage quality, directly threatening food security and livelihoods amid intensifying climate-driven water scarcity. Enhancing drought resilience in alfalfa is thus a paramount agricultural imperative. This study elucidates the synergistic interactions between genetic factors (genotypes) and management interventions (biostimulants) on drought tolerance, productivity, and quality traits effects underexplored in alfalfa systems despite individual studies on each. The research therefore aims to identify cultivar-biostimulant combinations that can sustain performance under water-limited conditions. The field experiment was conducted on fertile chernozem soil at the University of Debrecen's experimental garden, Hungary, across the 2023 – 2025 cropping seasons. The study involved two factors: (1). Two different alfalfa genotypes, namely Magor variegated alfalfa (*Medicago x varia* Martyn) and Hunor-40 alfalfa (*Medicago sativa* L). and (2) four biostimulant levels: control (T0), biostimulant containing Si, MTU[®] Pidolic acid (T1), Tricho Immun + Ino Green (T2) and Tricho Immun (T3). Drought severity was quantified via the Pálfaí Drought Index, classifying 2023 as mild, 2024 as moderate, and 2025 as a medium drought. Key metrics included plant height, NDVI, net photosynthetic rate, dry matter yield, forage quality, and protein yield were measured. Our results showed that genotype x biostimulant interactions significantly ($P < 0.05$), enhanced net photosynthesis rate and DMY, growth (height), NDVI, and quality traits across seasons. Under mild drought, net photosynthetic rates in Magor increased by 71% by T2, 86% with T1 under moderate drought, and 21% with T1 under medium drought. Critically, Magor + T2 (Tricho Immun + Ino Green) consistently maximized DMY at 37,592 kg ha⁻¹ under mild, 46,711 kg ha⁻¹ under moderate, and 43,301 kg ha⁻¹ under medium drought, outyielding Hunor across cropping seasons under drought severities. Although T1-T3 DMYs were statistically comparable except against the control, these gains underscore Magor + T2's superiority. Main effects further bolstered this: biostimulants boosted height between 12 – 29%, NDVI from 4 - 11%, and protein yield from 9.5 - 42%, whereas genotypes enhanced height from 9 - 13%, NDVI from 3 - 14%, and protein yield from 12–26% under drought stress conditions. These results compellingly demonstrate that integrating targeted biostimulants with drought-resilient genotypes like Magor substantially elevates biomass productivity, forage quality, and protein yield under varying water deficits validating biostimulants as potent resilience tools and genotype selection as essential for climate-adaptive

management. While improvements in physiological and yield traits are evident, future research should optimize application timing, irrigation regimes, and genotype diversity across agroecosystems to maximize scalability.

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10. PUBLICATIONS



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List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (2)

1. **Appiah, E. A.**, Kuunya, R., Kutasy, E.: Dynamics of alfalfa production in Hungary: Changes in harvested area, yield, and total production over 30 years (1990-2024).
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2. **Appiah, E. A.**, Virág, I. C., Kutasy, E.: Biostimulant induce growth, chlorophyll content and fresh herbage yield of alfalfa (*Medicago sativa* L.) and variegated alfalfa (*Medicago x varia* Martyn) plant.
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DOI: <http://dx.doi.org/10.34101/actaagrar/1/13552>

Foreign language scientific articles in international journals (1)

3. **Appiah, E. A.**, Balláné Kovács, A., Ocwa, A., Csajbók, J., Kutasy, E.: Enhancing Alfalfa (*Medicago sativa* L.) Productivity: Exploring the Significance of Potassium Nutrition.
Agronomy-Basel. 14 (8), 1-20, 2024. EISSN: 2073-4395.
DOI: <http://dx.doi.org/10.3390/agronomy14081806>
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Foreign language conference proceedings (1)

4. **Appiah, E. A.**, Kutasy, E.: Sustainable strategies for boosting alfalfa yield and quality under drought conditions.
RES J AGR SCI. 58 (1), 13-19, 2026. EISSN: 2668-926X.
DOI: <http://doi.org/10.59463/RJAS.2026.1.02>

Foreign language abstracts (6)

5. **Appiah, E. A.**, Kutasy, E.: Sustainable Strategies for Enhancing Growth Performance of Alfalfa Under Moderate Drought Stress using Biostimulant and Nutrient Management.
In: XXIX. Tavasz Szél Konferencia 2026: Absztrakt kötet. Szerk.: Molnár Dániel, Molnár Dóra, DOSZ, Budapest, 69, 2026. ISBN: 9786156457967





6. **Appiah, E. A.**, Kutasy, E.: Biostimulant-Induced Modulation of Photosynthetic Efficiency in Alfalfa (*Medicago sativa* L.).
In: The 5th International Electronic Conference on Agronomy 15-18 December 2025 | Online
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7. **Appiah, E. A.**, Kutasy, E.: Boosting Alfalfa productivity: The Impact of Tricho Immun supplemented with Foliar Fertilizer on Amino Acids and Protein Concentration.
In: Scientific Conference of PhD Students of FAFR, FBFS and FHLE SUA in Nitra with international participation: Book of abstracts. Eds.: Martina Fikselová, Veronika Štefúnová, Andrea Matušková, Dominik Holý, Zuzana Schubertová, Slovak University of Agriculture in Nitra, Nitra, 24, 2024. ISBN: 9788055228310
8. **Appiah, E. A.**, Kutasy, E.: Greener alfalfa fields: unlocking dry matter potential with biostimulants and foliar nutrition.
In: 22nd Wellmann International Scientific Conference. Eds.: Dávid Kóteles; Flórián Kovács; Ingrid Melinda Gyalai; László Beier; Szilárd Czóbel, University of Szeged Faculty of Agriculture, Hódmezővásárhely, Hungary, 25, 2025. ISBN: 9789636880446
9. **Appiah, E. A.**, Kutasy, E.: Comparative Yield Response of Two Alfalfa Cultivars to Biosimulant Supply.
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10. **Appiah, E. A.**, Kutasy, E.: Enhancing Biomass Yield and Elemental Composition of Alfalfa (*Medicago sativa* L.) Plants via Biostimulant Application Combined with Foliar Fertilization.
In: 11th International Symposium on Recent Advances in Food Analysis : Book of abstracts. Eds.: Jana Pulkrabová; Monika Tomaniová; Stefan van Leeuwen; Michele Suman; Michel Nielen, Jana Hajšlová, University of Chemistry and Technology, Prague Technická 5 166 28 Praha 6 Czech Republic, 301, 2024. ISBN: 9788075922687

List of other publications

Foreign language scientific articles in international journals (6)

11. Bytyqi, B., Forgács, F., Melash, A. A., Virág, I. C., Csajbók, J., **Appiah, E. A.**, Kutasy, E.: Foliar application of silicon and sulfur modifies grain mineral composition of spring oats (*Avena sativa* L.) under contrasting seasonal drought conditions.
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12. **Appiah, E. A.**, Nyandi, M. S., Ocwa, A., Duodu, E. J., Kutasy, E.: Role of Biostimulants in Sustainable Soybean (*Glycine max* L.) Production: A Systematic Review.
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11. APPENDICES

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12. DECLARATIONS

STATEMENT

I wrote this thesis in the framework of the University of Debrecen Kálmán Kerpely Doctoral School for the purpose of obtaining a doctoral degree (Ph.D.) at the University of Debrecen.

Debrecen, 24 September 2026



.....
signature of the candidate
Ebenezer Ayew Appiah

STATEMENT

I hereby certify that the doctoral candidate Ebenezer Ayew Appiah has carried out his work under my supervision within the framework of the above-mentioned Doctoral School between 2022-2026. The candidate has made a decisive contribution to the results of the thesis through his independent creative work, and the thesis is the candidate's independent work. I recommend that the thesis be accepted.

Debrecen, 24 September 2026



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signature of the supervisor
Dr. Erika Tünde Kutasy