

**Thesis of Doctoral (PhD) Dissertation**

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

**Ebenezer Ayew Appiah**  
PhD Candidate

Supervisor:

**Dr. Erika Tünde Kutasy**  
Associate Professor



**UNIVERSITY OF DEBRECEN**

**Kálmán Kerpely Doctoral School of Crop Production and Horticultural  
Sciences**

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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 (Jabesaa and Bekele, 2021). 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). 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). In alfalfa, drought not only reduces biomass accumulation but also impairs plant persistence, stand longevity, and nutritive value (Putnam, 2021; Appiah et al., 2024). The physiological mechanisms underlying drought-induced damage include reduced photosynthetic efficiency, limited root water uptake, oxidative stress, and impairment of nitrogen fixation processes (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). 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; 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). 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. MATERIAL AND METHODS

### 2.1 Description of the experimental site

A three-year field experiment was conducted at the research garden of the University of Debrecen, 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.

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 1**). 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 1**).

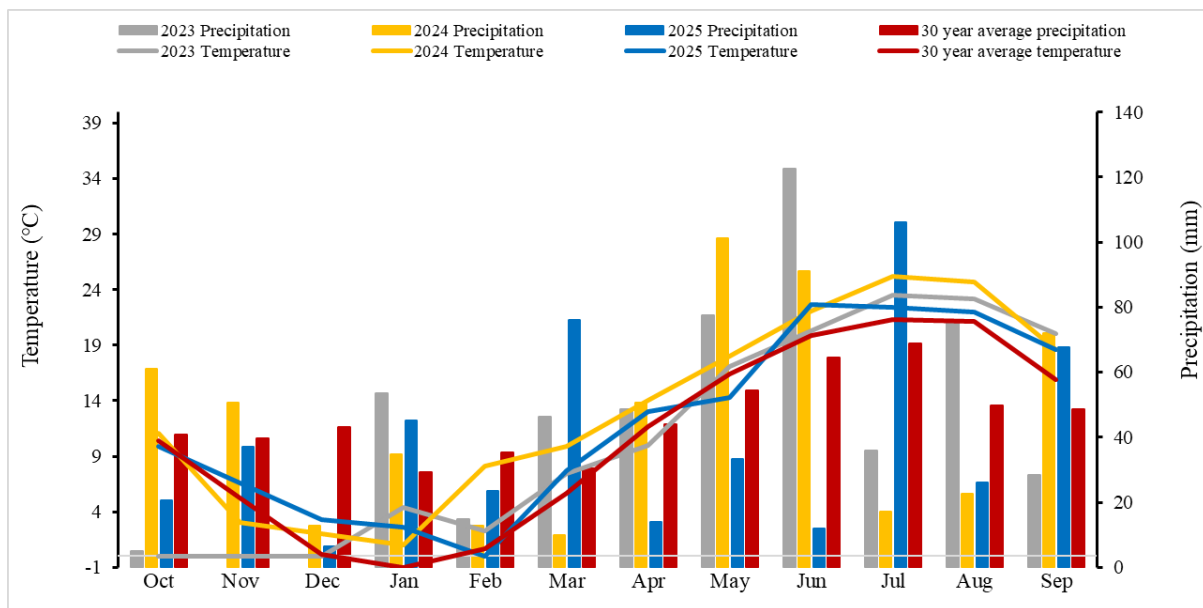


Figure 1: Meteorological data of the University of Debrecen experimental garden during the three-year study period (2023-2025) and the average for the past 30 years (Debrecen)  
*Source: Data from the measurement program of the Agrometeorological Observatory (AMO) of the Centre for Precision Farming R&D Services, University of Debrecen, Hungary*

## 2.2 Experimental design and treatment

The experiment was arranged in a randomized, completely blocked design replicated three times. A plot size of (3 x 4 m = 12 m<sup>2</sup>) was used with 1 m allays between plots and 2 m between replications. Two distinct different alfalfa genotypes, namely Magor variegated alfalfa (*Medicago x varia* Martyn) and Hunor-40 alfalfa (*Medicago sativa* L) obtained from the DE AKIT Nyíregyháza Research Institute was investigated. These alfalfa genotypes were sown in March, 2022, after maize forecrop under four treatment levels specifically, biostimulant containing Si, MTU<sup>®</sup> Pidolic acid (T1), *Tricho Immun* + Ino Green (T2), *Tricho Immun* alone (T3) and a control (T0). *Tricho Immun* was applied a day after cutting into the soil. In contrast, biostimulants containing MTU<sup>®</sup>, 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.

## 2.3 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 1**). 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 5<sup>th</sup> harvest, no treatments or measurements were carried out.

Table 1: Table 1: Harvesting period across growing season (Debrecen, 2023-2025)

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

Note: 1<sup>st</sup> phase: from 1<sup>st</sup> to 2<sup>nd</sup> cutting; 2<sup>nd</sup> phase: from 2<sup>nd</sup> to 3<sup>rd</sup> cutting; 3<sup>rd</sup> phase: from 3<sup>rd</sup> to 4<sup>th</sup> cutting, 4<sup>th</sup> phase: from 4<sup>th</sup> to 5<sup>th</sup> cutting

## 2.4 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.

### 2.4.1 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.

### 2.4.2 Net photosynthetic rate

Net photosynthetic rate was measured on intact leaves using a LI-6800 portable photosynthesis system (LI-COR, Lincoln, NE, USA) at two-week intervals. The instrument is equipped with two high-precision infrared gas analyzers that determine the mole fractions of CO<sub>2</sub> and H<sub>2</sub>O in the air. Photosynthetically active radiation in the chamber was maintained at 1500  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , with a spectral composition of 90% red light (625 nm) and 10% blue light (475 nm). The chamber CO<sub>2</sub> concentration was maintained at 400  $\mu\text{mol mol}^{-1}$ . Measurements were performed on light-adapted leaves, with six readings taken per leaf on two plants from each plot.

### 2.4.3 Dry matter 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 ( $\text{kg m}^{-2}$ ). These fresh weights were then multiplied by 10,000 to convert from  $\text{kg m}^{-2}$  to  $\text{kg ha}^{-1}$ . 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

$$\text{Dry matter (DM \%)} = \frac{\text{Dry weight (g)}}{\text{Fresh weight (g)}} \times 100 \dots\dots\dots (1)$$

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.

$$\text{Dry matter yield (kg ha}^{-1}\text{)} = \text{Fresh biomass yield (kg ha}^{-1}\text{)} \times \text{Dry matter (DM \%)} \dots\dots\dots (2)$$

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 2. 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).

$$\text{Standardised Yield (15\% moisture)} = \frac{\text{Dry matter yield (kg DM ha}^{-1}\text{)}}{0.85} \dots\dots\dots (3)$$

#### 2.4.4 Determination of protein yield

Protein yield was calculated using the formula 'protein yield (kg ha<sup>-1</sup>) = CP x DMY .....(4) where CP = crude protein content in percentage and DMY = dry matter yield in kg ha<sup>-1</sup>

#### 2.4.5 Quality traits determination

Plant samples harvested during the 2023–2025 cropping seasons were analyzed at the University of Debrecen. Dry matter content was determined by drying 500 g of fresh biomass at 103°C until reaching constant weight. Dried samples were ground to pass through a 1 mm mesh sieve. Nitrogen content was analyzed using the Kjeldahl method, with protein calculated as N × 6.25. Total sugars and carbohydrates were determined according to Thomas and Dutcher. Fat content was measured via the Randall-modified Soxhlet method: Fat content % =  $\frac{M_2 - M_1}{M_3 - M_1} \times 100$ , where  $M_1$  is the weight of the extraction crucible,  $M_2$  is the weight of the crucible plus dried petroleum ether extract, and  $M_3$  is the weight of the crucible plus the test sample. For saturated fatty acid analysis, total lipids were extracted, saponified with NaOH:MeOH at 80°C for 10 min, and unsaponifiable matter was extracted with hexane. The aqueous layer was then acidified with H<sub>2</sub>SO<sub>4</sub>, extracted with hexane, and derivatized with BF<sub>3</sub>:MeOH at 80°C for 30 min. Finally, the product was salted out with NaCl, dried with sodium sulfate, and analyzed by gas chromatography

#### 2.5 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. Data on precipitation and temperature on a daily basis were obtained from the Observatory Centre to calculate the Pálfai Drought Index (PaDI) to measure the water scarcity of our experiment site based on the Pálfai and Herceg (2012) the PaDi index following these steps below:

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

**PaDI<sub>0</sub>** represent the base drought index, which it is expressed in (°C 100 mm<sup>-1</sup>).

**T<sub>i</sub>** represent the average monthly temperature for five months in a row April to August (°C).

**P<sub>i</sub>** referes to the total monthly precipitation measured from October to September (mm).

$w_i$  represent monthly weighting factor to calculate the PaDi index. The  $w_i$  values are as followed for calculated months: 0.1 for October; 0.4 for November – December; 0.5 for January – April; 0.8 for May; 1.2 for June; 1.6 for July; 0.9 for August and 0.1 for September.

c present a constant value which is equal to 10 mm.

The drought index corrections factors ( $k_1, k_2, k_3$ ) are calculated as it is presented bellow:

a)  $k_1$  represent temperature correction factor

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

$T_{jun} + T_{jul} + T_{aug}$  presenting annual temperature average for the June-August (°C).

$\bar{T}_{jun}, \bar{T}_{jul}, \bar{T}_{aug}$  referring to 30 years average temperature (1991-2020) for June-August for period in (°C).

b)  $k_2$  represents correction factor for precipitation

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

$\bar{P}_{summer}^{min}$  referring to the lowest value from 30-years (1991-2020) precipitation sum of three summer months (June, July, August) in (mm).

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

c)  $k_3$  refereing the correction factor, for the precipitation circumstances of the previous period

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

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

$\bar{P}$  referring to mean of multi-annual precipitation sum for period October-September in (mm).

$\bar{P}_{36m}$  presenting mean precipitation sum for period October-September for previous three years in (mm).

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

$P_i$  referring to monthly precipitation sum in (mm).

PaDi index calculation in ( $^{\circ}\text{C } 100 \text{ mm}^{-1}$ )

$$PaDI = PaDI_0 * k_1 * k_2 * k_3$$

### 3.6 Data Analysis

The factors (biostimulants and alfalfa genotype) 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. Before 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 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.

### 3 RESULTS AND DISCUSSION

#### 3.1 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 2**).

Table 2: The calculated Palfai Drought Index during three growing seasons in the experimental site in Debrecen (2023-2025).

| Year | $P_{36 \text{ month}}$<br>mm | Monthly average P for previous 36 month mm | Sum of precipitation n April-August mm | $\text{MIN}(P_{\text{jun}}, P_{\text{jul}}, P_{\text{aug}})$<br>mm | $\text{PaDI}_0$<br>$^{\circ}\text{C } 100 \text{ mm}^{-1}$ | $\text{PaDI}$<br>$^{\circ}\text{C } 100 \text{ mm}^{-1}$ | 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.

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

**Table 3** presents the complex interactive effects of biostimulant application (B), genotype (G), and crop year/season (Y) on plant height (PH) of alfalfa at 14, 21, and 28 days after harvest (DAH) across four growth phases under contrasting drought stress conditions (2023–2025).

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. 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.

Table 3: 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 |            |           |           |

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.

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). 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.

### **3.3 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 4**). 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. 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. 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). 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 cues (Appiah et al., 2024). 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 4: 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 $\eta^2$ |
|-------------------|-----|-------|---------|------------------|
| 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  $\eta^2$  = partial eta square

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

The total dry matter yield of alfalfa was significantly modulated by the interplay of biostimulant treatments, genotypic differences, and environmental stress conditions across three experimental years (**Figure 2**). Across all three experimental years, both genotypes examined *Medicago x varia* Martyn (variegated alfalfa, Magor) and *Medicago sativa*

demonstrated a consistent improvement in TDMY following biostimulant application compared to untreated controls, although the magnitude of this response varied by treatment intensity and genetic background.

The performance of the genotypes varied markedly across the study period. In 2023, baseline yields were relatively low, with the minimum observed in untreated plots: 34,111 kg ha<sup>-1</sup> for Magor and 29,176 kg ha<sup>-1</sup> for Hunor. Biostimulant application facilitated significant yield recovery; the T2 treatment proved particularly effective, eliciting peak yields of 37,592 kg ha<sup>-1</sup> in Magor and 35,314 kg ha<sup>-1</sup> in Hunor under the T3 treatment. The 2024 season saw a general increase in DMY across all experimental plots, likely reflecting favorable conditions. Control plots yielded 35,808 kg ha<sup>-1</sup> and 33,024 kg ha<sup>-1</sup>. Notably, the T2 treatment achieved the study-wide peak, reaching 46,711 kg ha<sup>-1</sup> in Magor and 42,828 kg ha<sup>-1</sup> in Hunor. By 2025, however, yields declined across all treatments, reflecting increased severity of drought stress. Despite this, biostimulants provided a crucial buffering effect: the T2 treatment maintained the highest TDMY for Magor (43,301 kg ha<sup>-1</sup>), whereas Hunor showed a more moderate response with a peak yield of 32,551 kg ha<sup>-1</sup> under T2. In contrast, the lowest yields were recorded in the control plots, with 29,045 kg ha<sup>-1</sup> for Magor and 24,935 kg ha<sup>-1</sup> for Hunor.

Throughout the study, *Medicago x varia* Martyn consistently outperformed *Medicago sativa* across all treatments and years. This superior performance suggests that Magor possesses stronger inherent drought tolerance or a more favorable physiological responsiveness to exogenous stress-mitigating applications. The yield advantage of Magor was particularly pronounced under the T2 treatment, suggesting a positive genotype × biostimulant interaction where the genetic background amplifies the efficacy of the treatment. While Hunor exhibited lower absolute yields, its consistent improvements under biostimulant application underscore its capacity to benefit from stress-ameliorating strategies, even under suboptimal conditions. Overall, the data demonstrates that biostimulants substantially enhance the productivity of drought-stressed alfalfa. Among the tested treatments, T2 consistently outperformed other treatments, resulting in an overall mean yield increase of 27% over untreated plots, while T3 and T1 provided increments of 23% and 18%, respectively. These substantial yield gains suggest that biostimulants mitigate drought-induced biomass reduction likely through mechanisms such as improved physiological resilience, enhanced nutrient uptake, or the stimulation of stress-adaptive metabolic pathways. These findings align with established literature demonstrating that drought stress significantly compromises canopy greenness, vegetative development, and biomass accumulation in forage crops (Liu et al., 2018)

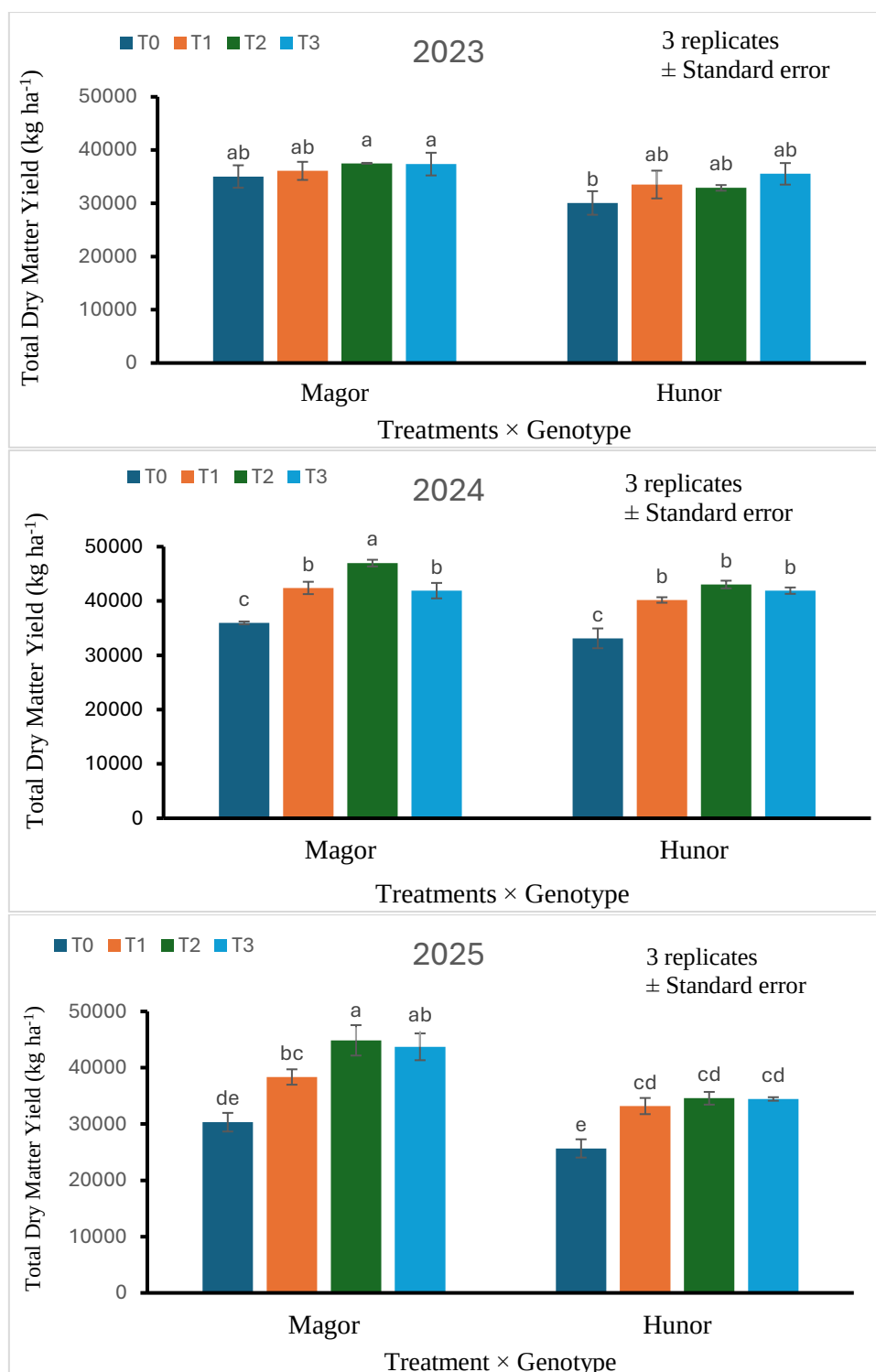


Figure 2: Effect of biostimulant treatments and genotype variability on total dry matter yield ( $\text{kg ha}^{-1}$ ) of alfalfa crop during the three cropping seasons (Debrecen. 2023–2025).

*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  $p = 5\%$ .*

Furthermore, the yield increments observed here mirror reports from a three-year trial by Marinova et al. (2023) who noted a 5% increase in alfalfa DMY using vermicompost-derived

biostimulants, alongside other studies reporting up to 30% yield increases with seaweed-based treatments. The robustness of the response across diverse climatic years strongly suggests that these biostimulant treatments serve as a viable buffer against drought-induced productivity losses in alfalfa production systems.

### 3.5 Complex evaluation of biostimulant treatment, genotype, year and their interactions on protein yield alfalfa under contrasting drought condition

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 5**). 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. 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 5: 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 $\eta^2$ |
|---------------------|----|--------|-------|------------------|
| 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  $\eta^2$  = partial eta square

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

The correlation analysis summarized in **Table 6** 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 under contrasting drought condition across three cropping seasons. TDMY exhibited strong positive correlations with plant height, particularly at 21 DAH ( $r = 0.639$ ,  $p < 0.001$ ) and 28 DAH ( $r = 0.553$ ,  $p < 0.001$ ), and vegetative indices, highlighting that late vegetative growth and canopy development are critical for final biomass accumulation. These findings demonstrated that plant stature could be a reliable indicator of forage productivity in perennial legumes, and confirm that NDVI effectively captures the severity of drought-induced canopy decline. In contrast, moderate negative correlations were observed between TDMY, NDVI, and NPR. Although photosynthesis is essential for carbon assimilation, instantaneous gas exchange measurements often decouple from final yield under water-limited conditions, where plants may prioritize stomatal regulation and altered carbon allocation over long-term growth. In addition, The drought index (PaDI value) exhibited consistent negative correlations with plant height at 14 days after harvest, NDVI from 14 to 28 DAH, and total dry matter yield, with R-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 total dry matter yield represents the cumulative impact of drought on productivity. Furthermore, NPR values in drought-stressed plants may reflect reduced canopy self-shading rather than increased productivity, as smaller canopies often exhibit higher leaf-level photosynthesis despite lower total biomass. Inter-trait correlations revealed consistent patterns: vertical growth was stable, with height variables across all stages showing strong positive associations. Similarly, vegetation indices were highly intercorrelated and positively linked to plant height, reinforcing the premise that increased canopy vigor is a key driver of structural development. Collectively, these results indicate that canopy-level morphological and spectral traits are more robust determinants of final biomass under drought stress than instantaneous physiological measurements, which provide only short-term snapshots of plant stress responses.

Table 6: 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)*

|                    | P <sub>03-07</sub> | PaDI   | PH at14DAH | PH at21DAH | PH at28DAH | NDVI at14DAH | NDVI at21DAH | NDVI at28DAH | NPR at14DAH | NPR at28DAH | TDMY |
|--------------------|--------------------|--------|------------|------------|------------|--------------|--------------|--------------|-------------|-------------|------|
| P <sub>03-07</sub> | -                  |        |            |            |            |              |              |              |             |             |      |
| 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*

### **3.7 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)**

We used Principal Component Analysis to examine how various biostimulant treatments affected the yield, growth, physiological, and biochemical traits of alfalfa over three growing seasons under contrasting drought condition. The PCA results indicate that these treatments had a significant impact on plant performance (**Figure 3**). The specific groupings show that treatments T2 and T3 noticeably altered the plants' growth and biochemical profiles, leading to higher overall yields. The first two principal components explained 63.5% of the total variance, with PC1 accounting for 37.7% and PC2 for 25.8%. There was a clear separation along PC1, particularly between the control group and the biostimulant treatments. The control group (T0) aligned more with traits like total carbohydrates and NDVI at 14 DAH, indicating that untreated plants tended to accumulate more carbohydrates early in the season but lagged in biomass and structural development. In contrast, T2 was associated with traits linked to higher productivity, including plant height (at 21 and 28 DAH), NDVI at 28 DAH, fibre content, total sugar, and total dry matter yield, confirming its effectiveness in promoting biomass. T3 showed a similar pattern but had more variability and a smaller impact on these productivity traits compared to T2. T1 fell in the middle, showing intermediate responses that overlapped with the other treatments. Overall, variables such as plant height, NDVI at 28 DAH, and total dry matter yield were strongly and positively correlated with one another. Conversely, total carbohydrates and NDVI at 14 DAH had an inverse relationship with these productivity traits. The clear distinction between treatment groups confirms that the biostimulants caused meaningful shifts in plant traits, with T2 and T3 significantly boosting yield and growth, whereas the control was mainly linked to early-stage physiological traits.

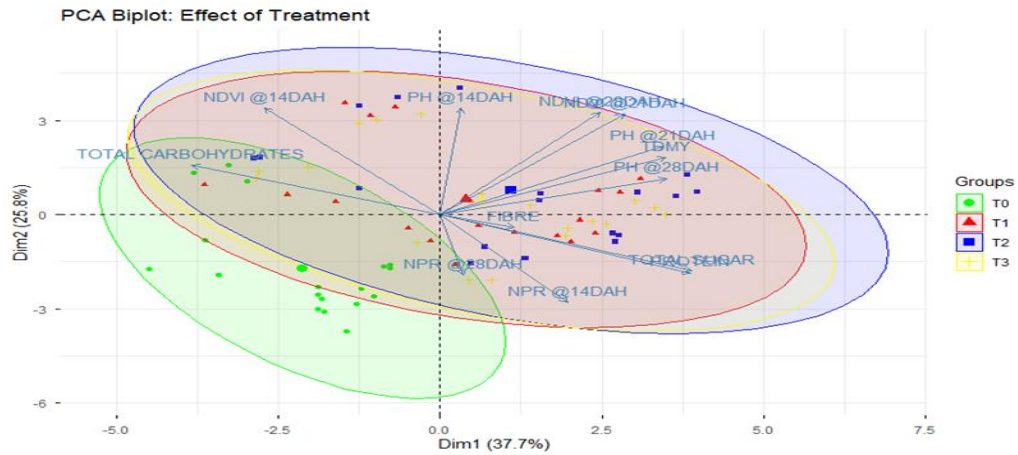


Figure 3: The principal component analysis biplot illustrating the effect of biostimulant treatments on the growth, physiological traits, biochemical and total dry matter yield of alfalfa. The first principal component explain 37.7% of variance whereas the second principal component contributes 25.8% making 63.5% of the total variability. (Debrecen, 2023-2025).

#### 4. 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<sup>®</sup>, 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<sup>®</sup>, 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.

## 5. PRACTICAL UTILISATION OF RESULTS

1. Farmers should consider using biostimulants containing MTU<sup>®</sup>, 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<sup>®</sup>, 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.

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## 7. PUBLICATIONS



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Registry number: DEENK/616/2026.PL  
Subject: PhD Publication List

Candidate: Ebenezer Ayew Appiah  
Doctoral School: Kálmán Kerpely Doctoral School  
MTMT ID: 10104413

### 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.  
*Acta agrar. Debr.* 2024 (1), 19-25, 2024. ISSN: 2416-1640.  
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>  
IF: 3.4

#### 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. Tavaszi 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  
. Ed.: Oscar Vicente, MDPI, Switzerland, 28, 2025.
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 Hollý, 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 Köteles; 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.  
In: The 4th International Electronic Conference on Agronomy. Ed.: Jose David Flores-Felix, MDPI AG, Basel, 150, 2024.
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.  
*Plants-Basel*. 15 (2), 1-28, 2026. ISSN: 2223-7747.  
DOI: <http://dx.doi.org/10.3390/plants15020316>  
IF: 4.7 (2025)





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. *Sustainability*. 18 (2), 1-23, 2026. ISSN: 2071-1050.  
DOI: <http://dx.doi.org/10.3390/su18020636>  
IF: 4.1 (2025)
13. Nyandi, M. S., **Appiah, E. A.**, Pepó, P.: Harnessing chlorophyll and canopy reflectance indices relationship for grain yield, protein and starch content in maize cultivars under different nitrogen treatments. *Plant Soil Environ*. 71 (8), 524-532, 2025. ISSN: 1214-1178.  
DOI: <http://dx.doi.org/10.17221/633/2024-PSE>  
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14. Davis, V. B., **Appiah, E. A.**, Tarcali, G.: Sustainable Control Strategies for *Ustilago maydis* in Maize: Effects on Plant Growth and Yield. *Journal of environmental science and agricultural research*. 3 (6), 1-5, 2025. ISSN: 3029-0724.  
DOI: <http://dx.doi.org/10.61440/JESAR.2025.v3.107>
15. Alidu, S. M., **Appiah, E. A.**: Improving the Photosynthetic Efficiency and Productivity of Cowpea in Sub Saharan Africa: A Review. *Journal of Scientific Research & Reports*. 28, 96-111, 2022. ISSN: 2320-0227.  
DOI: <http://dx.doi.org/10.9734/jsrr/2022/v28i111707>
16. **Appiah, E. A.**, Kugbe, J. X., Rufai, A. M.: Maize Growth and Yield Response to Incremental Rates of Nitrogen in N-Depleted Lixisols in Northern Ghana. *International Journal of Plant & Soil Science*. 32, 16-30, 2020. ISSN: 2320-7035.  
DOI: <http://dx.doi.org/10.9734/ijpss/2020/v32i1830388>

**Total IF of journals (all publications): 14,7**

**Total IF of journals (publications related to the dissertation): 3,4**

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

28 September, 2026

