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Biostimulant induce growth, chlorophyll content and fresh herbage yield of alfalfa (*Medicago sativa* L.*)* and variegated alfalfa *(Medicago × varia* Martyn*)* plant

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SUMMARY

The use of biostimulants is associated with promoting plant growth by stimulating cell division and improving nutrient availability and uptake. A study was conducted at the University of Debrecen, Hungary, to examine the effect of biostimulants on alfalfa growth, chlorophyll content and fresh herbage yield. The experiment was arranged in a randomised complete block design with three biostimulant treatments plus control replicated three times. Data collected were subjected to analyses of variance using Genstat, where significantly different means were separated at a probability of 5% using the least significant difference. The findings show no different variation in plant height or chlorophyll content (SPAD) throughout the early stages of growth. Nonetheless, a notable impact was noted in the latter stages (28 days after biostimulant treatment application) on the growth of the alfalfa plant. Biostimulant treatments did not had effect on fresh yield for second through fourth cuts, but the fifth cut showed a significant effect, with T1 treatment recording the highest herbage yield of 19745 kg ha^{-1} followed by T2 (Tricho Immun plus Ino Green) and T3 (Tricho Immun), with yields of 19528 kg ha^{-1} and 17273 kg ha^{-1}, respectively, while the T0 (control) recorded the lowest herbage yield of 12060 kg ha^{-1}. However, the average mean yield indicated the application of biostimulants significantly increased fresh yield herbage by 20.5%. Correlation coefficient values suggested plant height at both 14 and 28 DAH (days after harvest) strongly correlated with fresh herbage yield (r = 0.7756 and 0.7455) which reflected in the increase in fresh herbage yield. Therefore, our results suggest that the use of biostimulants in alfalfa cultivation holds promise for improving growth and yield potential through their positive effects on chlorophyll content and the growth of alfalfa plant.

Keywords: *Alfalfa; variegated alfalfa; SPAD; Trichoderma spp; fresh herbage yield*

INTRODUCTION

Alfalfa (*Medicago sativa* L), is a perennial legume whose growth and nutritional value are essential to animal husbandry and sustainable agriculture. It is a useful forage crop that is widely used in animal feed (Latif et al., 2023) because of its well-established functions in animal nutrition, soil enhancement, and atmospheric nitrogen sequestration. It is a crucial part of sustainable crop rotations (El Moussaoui et al., 2023). In Hungary, alfalfa is considered one of the most important forage crops which plays a vital role in the livestock industry because of its high protein content and other nutritive values. Alfalfa is an important component of sustainable agricultural systems and a major source of income for most farming communities. Improving alfalfa productivity is of importance for sustainable agriculture and meeting ever-growing world population demands.

Improving crop productivity and its resilience to unfavourable conditions by the use of agrochemicals has thrived as a common practice amongst growers. Nonetheless, there are several detrimental effects on the environment linked to the increased usage of these products (Pandey and Diwan, 2018; Tyagi et al., 2022). As a result of these negative implications, researchers have turned their attention to innovative strategies that are eco-friendly and can optimize plant growth and increase crop yields while minimising the use of synthetic chemicals.

One such strategy that has garnered significant attention in recent years is the application of biostimulants, which are described by De Vasconcelos and Chaves (2019) as a diverse group of compounds that stimulate plant growth, improve nutrient uptake, and confer tolerance to environmental stress. These compounds are increasingly being integrated into production systems to modify physiological processes in plants to optimise productivity. Samuels et al. (2022) classify biostimulants into the following categories: humic, seaweed, beneficial microbes, plant extracts, inorganic, chitosan/biopolymers, and amino acids. Their vital function is to promote the growth of a plant's leaves, stems, and roots to make up for any nutrient deficiencies that may arise throughout the growing season due to different factors, including drought.

The use of biostimulants such as seaweed extract has been reported to impact growth and development positively (Abass et al., 2020; Hernández-Herrera et al., 2022). The major component of this extract is polysaccharide which possesses plant growth-promoting activities and is also known for championing plant defence response against diseases (fungal, bacteria pathogens) (Albrecht, 2019). In addition, seaweeds are known to have a beneficial effect on plants' ability to retain water. Applying these materials at the early growth stage enhances the vitality and vigour of the plant which helps them to withstand unfavourable weather conditions (Malik et al., 2020). Based on scientific evidence, the application of plant-based biostimulants promotes the growth response of radishes and tomatoes (Fiorentino et al., 2018; Raza et al., 2022) and increases the amount and quality of crop yield in addition to producing more chlorophyll (Puglisi et al., 2020).

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Trichoderma spp is considered a microbial plant biostimulant because of its main effects on photosynthesis and metabolic rates, possible action as a biocontrol agent (Kovács et al., 2021), and boost of increased nutrient uptake efficiency in plants (Abirami et al., 2022). Trichoderma enhances the plant's capacity to absorb nutrients, increase the roots' capacity to branch, and eventually increase the productivity of the plant (Abirami et al., 2022). Additionally, plants that have been infected by Trichoderma produce plant enzymes, antioxidants, and hormones that promote growth (auxin, gibberellins) and resistance to abiotic stress (Csótó et al., 2023). Godlewska and Ciepiela (2016) found that biostimulants containing plant hormones such as auxins and cytokinins had a beneficial effect on the feed value of forage grasses. Murawska et al. (2017) have revealed that biostimulants have a positive impact on the development of root systems, water retention capacity, chlorophyll content, and photosynthetic rate, all of which boost crop nutrient intake. In addition, *Trichoderma*-based biostimulants enhance the soil environment's quality, and growth and protect against disease infestation (Fiorentino et al., 2018; Gebashe et al., 2021) and stimulate growth and nitrogen assimilation (Ertani et al., 2013). Although the effect of biostimulants on several crops are well documented, information on their beneficial effect on alfalfa productivity is scanty therefore, this study seeks to investigate the effects of biostimulants on the growth, chlorophyll content (SPAD), and fresh herbage yield of alfalfa, to better understand the complex mechanisms underlying improved plant growth and its potential broader consequences for the agricultural sector.

MATERIALS AND METHODS

Description of the experimental site

A field experiment was conducted at the research garden of the University of Debrecen 'Hungary 'during the 2023 cropping season. The area 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 average temperature during the growing period was 10–23.5 °C and humidity was also 69–73%. The rainfall during the trial period was 503.2 mm. The record of the rainfall from April to September is presented in *Figure 1*. The soil chemical analysis revealed pH at the depth of 0–20 cm was moderately acidic but at 20–40 cm was slightly basic. Si, Na, organic carbon (OC), and Zn were all low. N, P and K were also moderate (*Table 1*).

Figure 1. **Average monthly rainfall from April to September 2023**

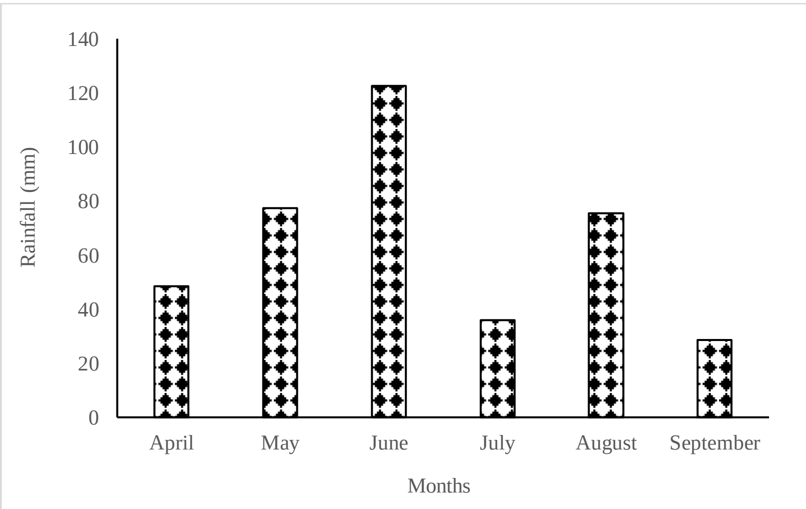

Table 1. **Soil chemical properties of the experimental site**

Parameters/Depth	0–20 (cm)	20–40 (cm)
pH (KCl)	6.93	7.46
OC (%)	2.89	2.87
P (mg kg^{-1})	1538	1149
N (mg kg^{-1})	82.2	53.4
K (mg kg^{-1})	638	586
Na (mg kg^{-1})	73.7	68.6
Si (mg kg^{-1})	35.8	28.0
Zn (mg kg^{-1})	2.77	2.24

*KCl= Potassium-chloride soluble

Experimental Design and Treatment

The experiment was arranged in a randomised complete blocked design consisting of four treatment levels replicated three times. A plot size of (3 m × 4 m = 12 m^2) with 1 m allays between plots and 2 m allays between replications were used.

In addition to the control, 3 treatments using a biostimulant, a growth promoter and a special foliage fertilizer were applied (*Table 2*).

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Table 2. **Treatment labels and levels used for the experiment**

Code	Treatment Levels	Dosage
T0	control (no application of treatment)	–
T1	Biostimulant containing MTU®, pidolic acid and Si	0.25 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

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 is the first internal Trichoderma. Composition: Patented strains of *Trichoderma afroharzianum* (TR04), *Trichoderma simmonsii* (TR05), 200 million (2x108) CFU g^{-1}

Tricho Immun in T2 and T3 was applied a day after cutting to the parcel, while T1 treatment and Ino Green (T2) were also applied a week after regrowth to the leaves.

Date of harvestings

Five separate harvests were made from the experiment on various dates. The first harvest took place on May 22, 2023; the second harvest took place on June 20, 2023; the third harvest took place on July 19, 2023; the fourth harvest took place on August 21, 2023; and the fifth harvest took place on September 26, 2023. 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 and measurements were carried out.

Data collection

Data were collected on plant height, leaf chlorophyll content (SPAD) and fresh herbage yield. Plant height was measured from the ground surface to the apex of the plant using the meter rule from six selected plants. The chlorophyll content of leaves was measured using SPAD-502 Plus (Konica Minolta Inc., Tokyo, Japan) from six randomly selected leaves per parcel. Data on plant heights were measured twice and chlorophyll content (SPAD) was measured three times for each cutting. Fresh herbage yield was measured on a plot basis, all the plants were harvested, gathered and weighed using a measuring scale.

Data analysis

Data gathered were analysed using one-way analysis of variance (ANOVA) using Genstat Statistical software package edition 18. Means were separated by at least a significance difference at a 5% probability level. DMRT was used to evaluate/rank the treatment performance from the highest to the least. Pearson correlation analysis was done to reveal the relationship among the parameters measured.

RESULTS

Plant height

The collected data showed that the application of the biostimulant had a distinct impact on the traits that were being assessed. Following the use of biostimulants, the data revealed no significant difference ($P > 0.05$) in height during the early growth stage (14 DAH). In contrast to T0, T2 measured the highest height in the range of 32 to 51 cm during the early growth stage across all cuttings (phases). Furthermore, a noteworthy variation in height was noted ($P < 0.05$) for all cuttings (phases) at 28 days following the application of the treatment. T1 achieved the highest height of 74.69 cm in comparison to T0, whereas T2 recorded the maximum height of 72, 68.65, and 64.3 cm at the first, second and third phases, respectively (*Table 3*).

Table 3. **Effect of biostimulants on plant height (cm) of alfalfa**

	1st phase		2nd phases		3rd phase		4th phases	
Treatment	**14 DAH**	**28 DAH**	**14 DAH**	**28 DAH**	**14 DAH**	**28 DAH**	**14 DAH**	**28 DAH**
T0	34.80^{a}	64.10^{a}	28.00^{a}	60.11^{a}	49.40^{a}	60.24^{a}	39.12^{a}	70.47^{a}
T1	38.95^{a}	68.70^{ab}	28.50^{a}	68.20^{b}	51.68^{a}	62.51^{ab}	40.45^{a}	74.69^{b}
T2	42.48^{a}	72.30^{b}	32.50^{a}	68.65^{b}	51.00^{a}	64.30^{b}	40.79^{a}	74.43^{ab}
T3	38.60^{a}	68.10^{ab}	31.00^{a}	67.20^{b}	50.00^{a}	63.79^{ab}	40.69^{a}	71.73^{ab}

*Means with similar or same letters are not significantly different at LSD (0.05), T0 = control, T1 = Treatment (containing MTU®, pidolic acid and Si), T2 = Tricho Immun + Ino Green, T3 = Tricho Immun, 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

Chlorophyll content

The chlorophyll levels evaluated after 14 DAH for all four cuts were not significantly affected ($P > 0.05$) by the application of the biostimulants. Still, *Table 4* shows that practically all treatment levels were statistically equivalent. In the first phase the chlorophyll content was significantly affected ($P > 0.05$) by the application of biostimulants, whereas in the second, third, and fourth phases the SPAD readings on 21 DAH showed no change. Nonetheless, the

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application of biostimulants had a substantial ($P < 0.05$) impact on the chlorophyll content measured on 28 DAH for the first, third, and fourth phases, but had no effect in the second phase. T2 and T3 had the greatest mean value of chlorophyll content (SPAD) in the fourth phase, with 63.98 and 64.17, respectively (*Table 4*).

Table 4. **Effect of biostimulants on chlorophyll content (SPAD) of alfalfa**

Treatments	14 DAH	21 DAH	28 DAH	Mean
		1st phase		
T0	44.62^{a}	49.27^{a}	50.79^{a}	48.22
T1	44.25^{a}	51.93^{ab}	53.02^{ab}	49.73
T2	44.54^{a}	53.08^{ab}	54.64^{b}	50.75
T3	45.08^{a}	52.45^{b}	53.68^{ab}	50.40
		2nd phase		
T0	45.91^{a}	48.18^{a}	57.86^{a}	50.65
T1	46.76^{a}	50.01^{a}	56.32^{a}	51.03
T2	45.00^{a}	49.40^{a}	57.72^{a}	50.70
T3	45.70^{a}	49.35^{a}	55.99^{a}	50.34
		3rd phase		
T0	47.76^{a}	49.98^{a}	50.63^{b}	49.45
T1	49.35^{a}	49.81^{a}	50.96^{b}	50.04
T2	47.28^{a}	49.49^{a}	47.40^{a}	48.05
T3	46.57^{a}	50.23^{a}	49.52^{ab}	48.77
		4th phase		
T0	46.11^{a}	54.96^{a}	58.85^{a}	53.30
T1	45.53^{a}	54.85^{a}	61.20^{ab}	53.86
T2	45.86^{a}	53.35^{a}	63.98^{b}	54.39
T3	45.86^{a}	53.81^{a}	64.17^{b}	54.61

*Means with similar or same letters are not significantly different at LSD (0.05), T0 = control, T1 = Treatment (containing MTU®, pidolic acid and Si), T2 = Tricho immun + Ino green, T3 = Tricho Immun, 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

Fresh herbage yield

Data on fresh herbage yield did not indicate a difference impact ($P > 0.05$) for the first to third phases after applying biostimulant treatments, but following the application of the biostimulant, a significantly altered ($P < 0.001$) fourth phase was noted. Regarding the applied treatment, fresh herbage yield generally increased. The first phase produced a far larger fresh herbage yield than the other phases. In comparison to plants that were not treated, the mean average showed that plants treated with biostimulant treatments had a substantial impact on fresh herbage yield. *Table 5* shows that T2 and T1 had the highest mean herbage yields, at 18389 and 18091 kg ha^{-1}, respectively, whereas T3 did not vary statistically from T2.

Table 5. **Effect of biostimulants on fresh herbage yield (kg ha^{-1}) of alfalfa**

Treatments	1st phase	2nd phase	3rd phase	4th phase	MEAN
T0	21839^{a}	15049^{a}	18039^{a}	12060^{a}	16746.75$^{\mathbf{a}}$
T1	20961^{a}	13803^{a}	17858^{a}	19745^{b}	18091.75$^{\mathbf{b}}$
T2	21978^{a}	14338^{a}	17714^{a}	19528^{b}	18389.50$^{\mathbf{b}}$
T3	21533^{a}	14938^{a}	17267^{a}	17273^{b}	17752.75$^{\mathbf{b}}$

*Means with similar or same letters are not significantly different at LSD (0.05,)T0 = control, T1 = Treatment (containing MTU®, pidolic acid and Si), T2 = Tricho immun + Ino green, T3 = Tricho Immun, 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.

The result of the correlation analysis between plant height, and SPAD readings measured and fresh herbage yield are presented in *Table 6*. The values from correlation coefficient (r) revealed that Plant height at both 14 and 28 DAH, and SPAD readings at 21 DAH were all positively correlated to fresh herbage yield. Correlation coefficient values which show the rate of association between SPAD, plant height and fresh herbage yield were noted decreasing at 28 DAH for plant height and SPAD at 14 DAH and 28 DAH respectively. A higher correlation coefficient value with a higher level of significance ($P < 0.001$) was observed at plant height at both 14 and 28 DAH. Plant height at 14 DAH strongly correlated with fresh herbage yield ($r = 0.7756$), followed by plant height at 28 DAH ($r = 0.7455$) respectively and SPAD at 21 DAH ($r = 0.2696$) (*Table 6*).

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Table 6. **Correlation analysis between plant height, SPAD readings (chlorophyll content) and herbage yield of alfalfa cultivated during the 2023 cropping season**

Parameters	PH_14DAH	PH_28DAH	SDR_14DAH	SDR_21 DAH	SDR_28 DAH	FHY
PH_14 DAH	–					
PH_28 DAH	0.8155	–				
SDR_14 DAH	-0.1611	-0.193	–			
SDR_21 DAH	0.3335	0.1956	0.1525	–		
SDR_28 DAH	0.1458	0.2711	-0.2641	0.316	–	
FHY	0.7756	0.7455	-0.0356	0.2696	-0.0045	–

PH = Plant height, SDR= SPAD readings, FHY = Fresh herbage yield, DAH =Days after harvest.

DISCUSSION

Beneficial properties of integrated biostimulants in crop productivity are their ability to enhance plant growth, water use efficiency (WUE), uptake of essential nutrients, chlorophyll, activation of plant secondary metabolism and photosynthesis. From our study, we observed that biostimulant did not affect plant height at the beginning of regrowth of the alfalfa plant, but plots treated with T1 treatment, Tricho plus Ino green (T2) and Tricho immun (T3) alone were slightly higher compare to control plot (T0). A similar result has been reported by Marinova et al. (2023). A significant increase in height was observed during the third week after the application of the biostimulants, this observation is in line with studies by Trawczyński (2020) and Mystkowska (2022) who reported a significant effect in potato height after the application of biostimulants, similar influence has been reported in soybean (Szparaga et al., 2018). T1 treatment and Tricho Immun plus Ino Green (T2) significantly improved the growth of alfalfa plants compared to the control (T0) but statistically not different from Tricho Immun alone, the increase in height could be due to components present in these biostimulants that may have promoted cell division and elongation, leading to increased growth of the alfalfa plants. This finding is in line with Alam et al., (2014) who stated applying biostimulants to plants modifies their morphological, physiological, and biochemical processes while increasing their capacity for absorbing nutrients. Additionally, a treatment combination of Tricho Immun plus Ino Green was found to enhance essential nutrient availability and uptake in the plant, resulting in vigorous growth and development of alfalfa reflecting in a significant increase in height at the third week of application. Elevated level of chlorophyll content in leaves promotes improved photosynthesis and carbohydrate synthesis leading to improvement in plant height, brunch count and final output (Nosheen et al., 2019).

The insignificance among biostimulant treatments on chlorophyll content (SPAD) during the early growth stage is in agreement with the study by Wadas and Dziugiel (2020) who did not observe the effect of biostimulants on chlorophyll content (SPAD). Application of biostimulant recorded the least chlorophyll content (SPAD) compared to the control plot but statistically not different (*Table 4*), similar results were noted by Vukelić et al. (2021) who noted a 13% least chlorophyll content in tomato plants under application of biostimulant. However, a significant influence on chlorophyll content (SPAD) was observed after 28 days after harvest under the application of treatments. These findings support the studies by (Mystkowska 2022). Trawczyński (2020) observed that the value of the SPAD index in potato leaves was impacted by biostimulant application. The study observed a general improvement in chlorophyll content (SPAD) during 28 days after harvest under application of treatments, this could be attributed to the stimulative effect of the biostimulant on chlorophyll synthesis and pigment accumulation. This study supports previous studies that have found biostimulants to increase chlorophyll content in plants by enhancing cell division and stimulating chlorophyll biosynthesis (Bulgari et al., 2015; Puglisi et al., 2020).

The results of this study indicate fresh herbage yield for the first phase was substantially higher than other phases, however, statistical analysis revealed no effect of the applied treatments from first to third phase (*Table 5*). The higher yield achieved under the (first phase) second cutting could be explained by the maximum rainfall during the growing period, which boosted plant growth and increased fresh herbage yield production. This observation is consistent with that of Retta et al. (1980), who found that when rainfall increased from 96 to 278 mm, alfalfa yield increased from 2890 to 17490 kg ha^{-1}. According to Hanson et al. (2007), crop evapotranspiration which is derived from rainfall or irrigation has a direct impact on alfalfa yields. However, the observed significant influence of biostimulant on herbage yield after cutting five (fourth phase) where herbage yield increased with application of biostimulants (*Table 5*), maximum herbage yield of 19745 kg ha^{-1} was obtained by T1 treatment, followed by T2 (Tricho Immun plus Ino Green) and T3 (Tricho Immun) with the yield of 19528 kg ha^{-1} and 17273 kg ha^{-1} respectively while minimum herbage yield of 12060 kg ha^{-1} obtained by the control, implies that application of biostimulants enhance nutrient availability and uptake in alfalfa plant and optimize vigorous growth development and herbage yield. This observation is in line with Godlewska and Ciepiela, (2020) who observed a significant increase in the biomass yield of red clover as affected by biostimulants application. The application of biostimulant treatments significantly increases fresh herbage yield by 20.5%, as indicated by the mean yield. The observed increase in fresh herbage yield is consistent with a study by De

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Beer et al. (2023) that found that the use of biostimulants, such as fulvic acid and bioflavonoids, improves N uptake efficiency and increases herbage quantity and quality. Peț (2008) reported that the application of biologically active products increased the dry matter yield of smooth brome. Stamatov (2020) observed that leaf fertilizers and biostimulators increased the yields of wheat and sunflower. Héctor-Ardisana (2021) presented preliminary results showing that the use of organic biostimulants resulted in similar or superior growth and yield compared to chemical fertilization in short-cycle crops. Correlation analysis further shows that fresh herbage yield is highly dependent on plant height, this is in agreement with Ramesh et al. (2002), who stated that the most important quantitative trait in crops, yielding capacity, is influenced by the development of other traits.

CONCLUSIONS

This study's findings demonstrated that the biostimulant treatments greatly enhanced fresh herbage yield, plant height, and chlorophyll content (SPAD). This study also found that the benefits of biostimulants are not always evident during the early stages of alfalfa growth, but rather become apparent later on in the plant's growth cycle following application. Application of T1 treatment (containing MTU®, pidolic acid and Si), Tricho Immun and Tricho Immun combined with Ino Green significantly impacted growth trait which resulted in higher fresh herbage yield compared to the control. The average mean fresh herbage yield of 18389 kg ha^{-1} for all cutting periods (phases) were noted with Tricho Immun plus Ino Green (T2) treatment combination, followed by T1 treatment (containing MTU®, pidolic acid and Si) and Tricho Immun (T3) with 18091 kg ha^{-1} and 17752 kg ha^{-1} respectively.

Application of biostimulant treatments positively increases fresh herbage yield by 20.5%. This suggests that biostimulants can be a valuable tool in improving growth and chlorophyll contents (SPAD), potentially increasing the fresh herbage yield of alfalfa, as they potentially enhance the yield, stimulating cell division, and improving nutrient availability and uptake. Therefore, the use of biostimulants in alfalfa cultivation holds promise for improving growth and yield potential through their positive effects on chlorophyll content and the growth of alfalfa plants.

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