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Effects of Different Fertilizer Types on Pigment Content and Some Stress Molecules in Perennial Ryegrass (*Lolium perenne* L.)

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ABSTRACT

Background: Perennial ryegrass (*Lolium perenne* L.) is a grass species known for its dark green color, fine texture and rapid germination in cool seasons. Its resilience to hot, dryand cold weather conditions makes it suitable for both warm and cool climates. Its high resistance to wear stress makes it an ideal plant for sports fields. The use of chemical fertilizers leads to the accumulation of toxic elements in the soil, reducing productivity and polluting water sources. In contrast, organic or biofertilizers improve soil structure, increase water retention capacityand enhance the effective use of plant nutrients. This results in high yields while ensuring environmentally friendly agricultural practices that protect human and animal health.

Methods: In this study, perennial ryegrass was treated with six different types of fertilizers (liquid seaweed fertilizer, microbial liquid organic fertilizer, organic liquid vermicompost, chicken manure, bat guanoand fertilizer with 32 micro trace elements) at four different fertilizer doses (2%, 4%, 6% and 8%). A control group with no fertilizer application was also included. The study investigated the effects of fertilizers on the plant's chlorophyll-a (mg/g), chlorophyll-b (mg/g), total chlorophyll (mg/g), chlorophyll a/b ratio, carotenoid (mg/g), proline (µmol/mL), MDA (Lipid peroxidation activity) (nmol/g), APX (Ascorbate Peroxidase) (EU/ml) and Catalase (EU/ml) levels.

Result: The results showed that chlorophyll a content ranged from 3.68 to 0.35, chlorophyll b from 2.07 to 0.22 and total chlorophyll from 5.75 to 0.56, while the chlorophyll a/b ratio was between 2.65 and 1.46. Carotenoid content ranged from 1.01 to 0.14and ascorbate peroxidase activity was measured between 29.02 and 0.67. Catalase activity varied from 175.80 to 11.54, proline content from 48.05 to 1.15 and lipid peroxidation (MDA) levels ranged from 1.50 to 0.36. These results demonstrate that different treatments have significant and wide-ranging effects on the physiological and biochemical parameters of plants.

Key words: MDA, Organic fertilizer, Perennial ryegrass, Proline, Pigment.

INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) is widely used in landscaping and grazing systems due to its adaptability to various environmental conditions and high nutritional value (Lin *et al*., 2016; Capstaff and Miller, 2018). Its resilience to stress conditions such as high temperature, droughtand cold makes it an ideal choice for landscape design and, particularly, for sports fields (Esfandyari *et al.,* 2020). The plant's high resistance to wear and traffic stress ensures the maintenance of turf quality despite heavy use of sports fields (Pornaro *et al.,* 2024). Turf areas, heavily managed to maintain uniform plant cover, provide crucial recreational spaces for urban populations' social, physicaland psychological health (Hedblom *et al*., 2017; Ignatieva *et al*., 2020; Cao and Kang, 2019; Evenson *et al*., 2019; Fuentes, 2021). The harmful effects of chemical fertilizers on soil and water have driven the shift toward organic and natural fertilizers for sustainable agriculture (Alori *et al*. 2017; Markov, 2015; Bozhanska and Naydenova, 2020). Sustainability focuses on conserving natural resources, reducing wasteand preventing pollution while meeting current and future needs (Emir and Yıldırım, 2024). Organic fertilizers enhance agricultural systems by boosting soil microbial diversity, structureand nutrient uptake (Kour *et al*. 2020; Dasgupta *et al*., 2021; Gautam *et al*., 2021; Sansinenea, 2021; Petkova *et al*., 2023; Essel *et al*., 2024). This study examined the effects of different organic fertilizers (liquid seaweed, microbial, vermicompost, chicken manure, bat guano) and one inorganic fertilizer (32 micro trace elements) on the pigment content and stress tolerance of perennial ryegrass. The goal is to identify the optimal fertilization strategy for sustainable turf management.

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MATERIALS AND METHODS

Plant material and experimental design

In March 2024, the growth of perennial ryegrass (*Lolium perenne* L.) under various fertilization conditions was studied in a greenhouse in Pazar, Rize, Turkey. Grass was grown in 16 cm diameter, 13 cm deep plastic pots filled with sterile peat (pH 6.0, 1.0 g/l fertilizer). The experiment used a factorial design with three replications. Seeding was done by broadcasting, followed by pressing and covering with peat (Fig 1). Thirty days after seeding, different fertilizer solutions (liquid seaweed, microbial organic, vermicompost, chicken manure, bat guanoand 32 micro trace elements) were applied at concentrations of 0, 2, 4, 6and 8%. Fertilizers were applied uniformly (500 ml) as aqueous solutions (Fig 2). The study measured

Fig 1: Emergence of plants from soil and vegetative growth stage.

Fig 2: Visible effects of different fertilizer types and dosages on plant growth, leaf color, leaf. size and overall plant health.

Chlorophyll-a, Chlorophyll-b, Total Chlorophyll, Chlorophyll a/b, Carotenoid, Proline, MDA (Lipid Peroxidation), APX (Ascorbate Peroxidase)and Catalase. Fertilizer contents are as follows (Fig 3).

Fertilizer types and contents

Liquid seaweed fertilizer

Organic Matter: 6% (w/w), pH: 3-5, Water-Soluble Potassium Oxide (K, O): 1% (w/w), Maximum EC (Electrical Conductivity): 41 dS/m (Fig 4).

Microbial liquid organic fertilizer

Names of live microorganisms: *Bacillus subtilis, Bacillus megaterium, Bacillus amyloliquefaciens*. Total Live Organism Count: 1×10^{8} CFU/ml (colony-forming units/ml) (Fig 4).

Organic liquid vermicompost

Organic Matter: 7%, Organic Nitrogen: 0.3%, Maximum EC (Electrical Conductivity): 0.5 dS/m, pH Range: 4.5-6.5 (Fig4).

Chicken manure

It contains 100% organic matter. It is derived from chicken manure extract. (Chicken Manure Extract) (Fig 4).

Bat Guano

Organic Matter: 25% (w/w), Organic Carbon: 7% (w/w), Organic Nitrogen (N): 2% (w/w), Water-Soluble Potassium Oxide (K, O): 5% (w/w), Total Phosphorus Pentoxide (P, O…): 1% (w/w), Total Humic+Fulvic Acid: 3% (w/w), pH: 5-7, Water-Soluble Calcium Oxide (CaO): 1% (w/w), Maximum EC (dS/m): 17% (w/w)

Fertilizer with 32 Micro trace elements

Total Nitrogen (N): 32% (w/w), Ammonium Nitrogen (N-NH„): 8% (w/w), Nitrate Nitrogen (N-NO*f*): 16% (w/w), Urea Nitrogen (N-NH,): 0.01% (w/w), Boron (B): 0.01% (w/w), Copper (Cu): 0.02% (w/w), Iron (Fe): 0.02% (w/w), Manganese (Mn): 0.01% (w/w), Molybdenum (Mo): 0.001% (w/w), Zinc (Zn): 0.002% (w/w)

Pigment analysis

The total chlorophyll and carotenoid content were determined using the Arnon, 1949 methodand the measured absorbance values were converted to chlorophyll

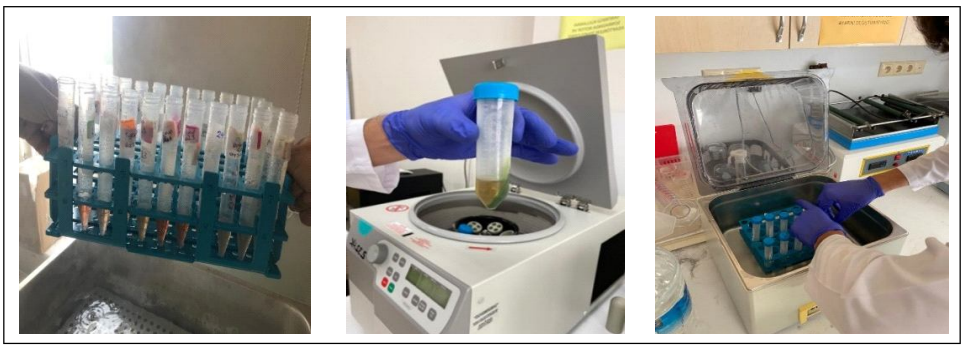

Fig 3: Quantitative determination of enzyme activities, pigmentsand other biochemical components.

a, chlorophyll b, total chlorophylland total carotenoid amounts using the formulas below (Fig 5):

Total chlorophyll (C) = [20.2 ×Δ645 + 8.02 × Δ663]× ml/1000
Chlorophyll a (Ca) = [12.7 × Δ663 - 2.69 × Δ645]×ml/100
Chlorophyll b (Cb) = [22.9 × Δ645 - 4.68 x Δ663]×ml/1000
Total Carotenoid = [1000 Δ470 - 1.90 × Ca - 63.14 × Cb] / 214] × ml/1000

Note: Absorbance measurements at 645 nm, 663 nmand 470 nm wavelengths were performed using a spectrophotometer.

Biochemical analysis

Proline (µmol/ml)

Proline analysis was conducted following Bates (1973). Leaf tissue (50 mg) was homogenized with 5 ml of 3% sulfosalicylic acid and centrifuged at 5000 g for 10 minutes. The supernatant was mixed with 2 ml acid-ninhydrin and 2 ml acetic acid, boiled for 1 hour at 100°C, cooledand extracted with 5 ml of toluene. The pink upper phase's absorbance was measured at 520 nmand proline content was calculated using a standard curve. Proline content is expressed as µmol/g fresh weight. (Şekil 5).

Lipid peroxidation activity (MDA) (nmol/g)

Using the method of Çakmak and Horst (1991), samples stored at -80°C were utilized. 0.2 g of plant tissue was homogenized with 1 ml of 0.1% TCA at +4°C for 10 minutes and centrifuged at 12,000 g for 20 minutes. The supernatant (250 µl) was mixed with 1 ml of 20% TCA containing 0.6% TBA, incubated at 95°C for 30 minutes, then rapidly cooled on ice. After centrifuging at 12,000 g for 10 minutes, absorbance was measured at 532 and 600 nm. Lipid peroxidation was calculated as Δ(532-600)/ 1.56×10u and expressed in nmol/L.

Ascorbate peroxidase (APX) (EU/ml)

APX activity was measured following Nakano and Asada (1981). Leaf tissue (0.5 g) was homogenized with extraction buffer (0.1 mM EDTA, 2 mM ascorbate, 2% PVPP, 50 mM PBS, pH 7) at +4°C and centrifuged at 14,000 rpm for 30 minutes. The supernatant was used for APX activity measurement, calculated as ΔAbs(290 nm)/(min × mg protein) and expressed in U/mg protein.

Catalase (EU/ml)

Catalase activity was determined according to Cho *et al*. (2000). A reaction mixture of phosphate buffer, H, O and water was prepared and incubated at 30°C for 3 minutes. Using the measurement buffer as a blank, absorbance was measured at 240 nm after adding 37.5 µl of supernatant to a quartz cuvette. Catalase activity was calculated as ΔAbs (240 nm)/(min × mg protein) and expressed as EU/mg protein.

Statistical analysis

Statistical analyses were performed using JMP software. Multiple comparisons were made using the Tukey test to determine the statistical significance of differences between groups.

RESULTS AND DISCUSSION

Chlorophyll a is a critical pigment that determines the photosynthetic capacity of plants, playing a vital role in plant growth and productivity. In this study, the effects of different treatments on chlorophyll a content were found to be significant ($p \leq 0.001$: ***). The I-2 treatment had the highest chlorophyll a content at 3.68, indicating that I-2 supports chlorophyll a synthesis in plants (Table 1). The O-6 (2.56) and M-6 (2.37) treatments also showed high chlorophyll a levels, but no statistically significant difference was found among the other treatments. The control group (0.54) and S-4 (0.35) treatments had the lowest chlorophyll a values, indicating a negative effect on its synthesis. The coefficient of variation (CV) was 38.37%, indicating moderate variability in chlorophyll a levels. Statistically significant differences in chlorophyll b levels were observed among treatments ($p \leq 0.001$: ***). The I-2 treatment had the highest chlorophyll b level (2.07), supporting chlorophyll b synthesis, while M-4 (1.38) and O-6 (1.10) also increased chlorophyll b content. In contrast, the control (0.29) and S-4 (0.22) treatments showed the lowest chlorophyll b levels. I-2 also had the

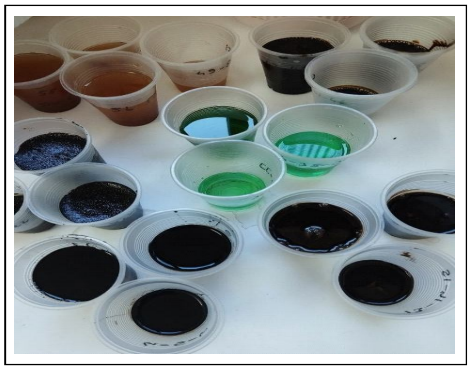

Fig 4: Fertilizer types and their liquid forms.

Fig 5: Process of preparing plant extracts: homogenization of samples and isolation of active components.

Table 1: Means and significance levels of Chlorophyll a, Chlorophyll b, Total Chlorophyll, Chlorophyll a/b, Carotenoid.

Application		Chlorophyll a	Application		Chlorophyll b	Application		Total Chlorophyll	Application		Chlorophyll a/b	Application		Carotenoid
İ-2	a	3.68	İ-2	a	2.07	İ-2	a	5.75	O-4	a	2.65	İ-2	a	1.01
O-6	ab	2.56	M-4	ab	1.38	O-6	b	3.66	T-4	a	2.63	O-6	ab	0.73
M-6	aabc	2.37	O-6	abc	1.10	M-6	bc	3.37	Y-4	ab	2.45	O-4	abc	0.69
O-4	aabcd	2.13	M-6	bcd	1.00	O-4	bcd	2,93	T-2	ab	2.43	M-6	bcd	0.59
T-4	bcde	1.99	O-4	bcde	0.80	M-4	bcd	2,89	T-6	ab	2.41	T-4	bcde	0.53
T-6	bcde	1.62	T-4	bcde	0.75	T-4	bcde	2,74	M-2	ab	2.38	T-2	bcde	0.51
T-2	bcde	1,58	T-6	cde	0.65	T-6	bcdef	2,27	M-6	ab	2.38	M-4	bcde	0.49
M-4	bcde	1.51	T-2	cde	0.65	T-2	bcdef	2,23	Y-2	ab	2.36	T-6	bcde	0.47
O-2	bcde	1.44	O-2	cde	0.59	O-2	bcdef	2,03	O-2	ab	2.34	O-2	bcde	0.38
Y-4	bcde	1.16	İ-4	cde	0.50	Y-4	cdef	1,64	O-6	ab	2.33	Y-4	bcde	0.37
Y-2	bcde	1.13	Y-2	cde	0.48	Y-2	cdef	1,61	S-2	ab	2.26	Y-2	bcde	0.35
İ-4	bcde	1.06	Y-4	cde	0.48	İ-4	cdef	1,56	S-6	ab	2.20	M-2	bcde	0.33
S-6	bcde	0.99	S-6	cde	0.45	S-6	cdef	1,44	İ-4	ab	2.06	S-6	bcde	0.33
M-2	bcde	0.97	M-2	cde	0.41	M-2	cdef	1,38	Y-8	ab	1.92	İ-4	cde	0.30
S-2	cde	0.85	S-2	de	0.37	S-2	def	1,22	Control	ab	1.87	S-2	cde	0.29
Y-8	de	0.65	Y-8	de	0.34	Y-8	def	0,98	Y-6	ab	1.86	Y-8	cde	0.26
Y-6	de	0.56	Y-6	de	0.29	Y-6	ef	0,85	İ-2	ab	1.78	Y-6	de	0.19
Control	de	0.54	Control	e	0.29	Control	ef	0,83	S-4	b	1.59	Control	de	0.18
S-4	e	0.35	S-4	e	0.22	S-4	f	0,56	M-4	b	1.46	S-4	e	0.14
CV (%)		38.37	CV (%)		33.99	CV (%)		31.15	CV (%)		14.72			30.62
Chlorophyll a		***	Chlorophyll b		***	Total chlorophyll		***	Chlorophyll a/b		***	Carotenoid		***

**p≤0.05: *, p ≤0.01: **, p≤0.001: *, n.s. (not significant), CV: Coefficient of variation value. KONTROL: Control, S-2, S-4, S-6: Liquid seaweed fertilizer (2%, 4%, 6%), M-2, M-4, M-6: Microbial liquid organic, Fertilizer (2%, 4%, 6%), O-2, O-4, O-6: Organic liquid vermicompost (2%, 4%, 6%), İ-2, İ-4: 32 Micro trace element fertilizer (2%, 4%), T-2, T-4, T-6: Chicken manure (2%, 4%, 6%), Y-2, Y-4, Y-6, Y-8: Bat Guano (2%, 4%, 6%, 8%).

Table 2: Means and significance levels of ascorbate peroxidase, catalase, proline, lipid peroxidase.

Application		Ascorbate peroxidase	Application		Catalase	Application		Proline	Application		Lipid peroxidase
O-2	a	29.02	S-2	a	175.80	T-4	a	48.05	T-2	a	1.50
Control	b	22.70	İ-2	b	172.96	T-6	b	45.94	Y-6	b	1.30
T-2	c	14.85	Y-8	c	163.42	O-6	c	38.65	O-4	c	1.25
M-2	d	12.22	T-6	d	110.39	İ-2	d	37.20	T-4	d	1.23
T-6	e	11.42	O-2	e	107.82	M-2	e	28.61	T-6	e	1.03
O-4	f	10.40	T-2	f	100.55	O-4	f	27.75	S-2	f	1.02
T-4	g	9.95	Y-2	g	97.14	M-4	g	20.78	O-2	g	0.99
M-6	h	9.41	Control	h	89.97	Control	h	19.17	İ-4	h	0.97
Y-6	ý	9.06	S-6	ý	88.09	S-6	ý	16.37	Y-8	h	0.97
S-6	j	8.67	T-4	j	86.17	İ-4	j	13.43	Y-4	ý	0.93
M-4	k	8.52	O-4	k	82.75	O-2	k	9.00	Y-2	j	0.91
S-2	l	7.14	O-6	l	52.91	T-2	k	8.47	Control	k	0.90
Ý-4	m	6.37	Y-6	m	46.16	Y-4	l	6.10	İ-2	l	0.86
Y-8	n	6.28	M-6	n	42.61	S-4	l	5.92	M-4	m	0.81
O-6	o	5.63	S-4	o	36.32	M-6	m	4.52	O-6	n	0.78
Y-2	p	4.59	İ-4	p	32.34	Y-6	n	3.95	S-6	o	0.75
S-4	q	3.54	M-4	q	22.57	S-2	o	3.10	M-2	p	0.67
Y-4	r	2.36	Y-4	r	16.12	Y-8	p	2.16	M-6	q	0.64
İ-2	s	0.67	M-2	s	11.54	Y-2	q	1.15	S-4	r	0.36
CV (%)		0.00	CV (%)		0.00	CV (%)		1.02	CV (%)		0.00
Ascorbate Peroxidase		***	Katalaz		***	Proline		***	Lipid Peroxidase		***

**p d≤0.05: *, p d≤0.01: **, p d≤0.001: *, n.s. (not significant), CV: Coefficient of variation value. KONTROL: Control, S-2, S-4, S-6: Liquid seaweed fertilizer (2%, 4%, 6%), M-2, M-4, M-6: Microbial liquid organic, Fertilizer (2%, 4%, 6%), O-2, O-4, O-6: Organic liquid vermicompost (2%, 4%, 6%), İ-2, İ-4: 32 Micro trace element fertilizer (2%, 4%), T-2, T-4, T-6: Chicken manure (2%, 4%, 6%), Y-2, Y-4, Y-6, Y-8: Bat Guano (2%, 4%, 6%, 8%).

highest total chlorophyll content (5.75), suggesting it promotes overall chlorophyll synthesis, while the control (0.83) and S-4 (0.56) treatments had the lowest total chlorophyll values (Table 1). Olszewska *et al*. (2022) reported that high nitrogen levels and biostimulants positively influenced chlorophyll synthesis in *Lolium perenne* L. These findings are consistent with our observations that certain treatments (*e.g*., I-2, O-6, M-6) increased chlorophyll a and b levels. Xu *et al.* 2021 aimed to evaluate the effects of high TN (Total Nitrogen) + TP (Total phosphorus) levels on seed germination, plant growth, antioxidant responseand N and P assimilation rates in perennial grass. Yuan *et al*. (2021) found that high TN + TP levels negatively impacted plant growth and photosynthesis, similar to our findings where the control group and S-4 treatments reduced chlorophyll levels due to adverse conditions. They also reported that silicon (Si) and potassium (K) applications improved chlorophyll synthesis in *Lolium perenne* L. under salt-alkaline stress, partially aligning with our results. The O-4 (2.65) and T-4 (2.63) treatments had the highest chlorophyll a/b ratios, suggesting improved light use efficiency. The control (1.87) showed a moderate ratio, while M-4 (1.46) and S-4 (1.59) had the lowest. The coefficient of variation (CV) was 14.72%, indicating low variability in the a/b ratio (Table 1). Carotenoid content varied significantly among treatments ($p \leq 0.001$: ***). The I-2 treatment had the highest carotenoid content (1.01), indicating a potential increase in antioxidant capacity, while O-6 (0.73) and O-4 (0.69) also had high levels. The control (0.18) and S-4 (0.14) treatments had the lowest carotenoid levels. The coefficient of variation (CV) was 30.62%, showing moderate variability (Table 1). Ascorbate peroxidase activity, an indicator of stress response, was highest in O-2 (29.02). The control (22.70) had moderate activityand I-2 (0.67) showed the lowest, suggesting a negative effect on stress response. The CV for ascorbate peroxidase activity was 0%, indicating no variability (Table 2).

Zuo *et al*. (2022) investigated how different nitrate levels affect the regrowth of *Lolium perenne* after grazing or defoliation, finding that medium nitrate levels enhanced photosynthetic pigments and efficiency. Our study aligns partially with these findings. Asghar *et al*. (2021) studied two wheat cultivars, showing that N+P fertilizer (80-60 kg/ha and 120-90 kg/ha) significantly improved yield-related traits and biochemical parameters. Foliar application of Proline and H_2O_2 boosted yield traits, with H_2O_2 enhancing ascorbate peroxidase (APX) and ascorbic acid (AsA) activities, while proline increased phenolic compounds. These results were similar to those of the O-2 fertilizer in our study, but not consistent with the I-2 treatment.

Catalase is a crucial antioxidant enzyme that helps plants detoxify harmful reactive oxygen species (ROS) generated under stress conditions, thereby protecting cells from oxidative damage. Increased catalase activity is often associated with enhanced stress tolerance in plants. Catalase activity also showed significant differences among the treatments ($p \leq 0.001$: *). S-2 treatment showed the highest catalase activity (175.80), with I-2 (172.96) and Y-8 (163.42) also high. The control group (89.97) had moderate activity, while M-2 had the lowest (11.54), possibly negatively affecting oxidative stress response. Proline content was highest in T-4 (48.05), indicating enhanced stress response; the control (19.17) was moderateand Y-2 had the lowest (1.15). Lipid peroxidase activity, indicating oxidative damage, was highest in T-2 (1.50), suggesting increased membrane damage; the control (0.90) was moderateand S-4 had the lowest (0.36). Overall, findings align with similar studies (Zuo *et al*., 2022; Asghar *et al*., 2021; Loudari *et al*., 2023; Safari *et al*. 2023; Wei *et al*., 2023; Aalipour *et al*. 2020; Azizi *et al*., 2021; Hossain *et al*., 2022; Zhang *et al*., 2023; Yildirim *et al*., 2023; Yıldırım *et al*., 2024). However, negative effects in some treatments highlight the need for further research on their impact on plant stress responses and antioxidant defenses.

CONCLUSION

This study examined the effects of different fertilizer treatments on chlorophyll, carotenoid, ascorbate peroxidase, catalase, prolineand lipid peroxidase levels in plants. The results revealed that the treatments had significant and statistically meaningful effects on the physiological and biochemical responses of the plants. The I-2 treatment supported chlorophyll synthesis in plants, resulting in the highest chlorophyll a and b levels. Total chlorophyll content also reached its highest level with the I-2 treatment, indicating an increase in the plant's overall photosynthetic capacity. The O-4 and T-4 treatments stood out in terms of the chlorophyll a/b ratio. In stress response ascorbate peroxidase activity, enhancing the plant's ability to cope with stress. The S-2 treatment had the highest catalase activity, supporting the plant's oxidative stress response. Proline accumulation was highest with the T-4 treatment, indicating increased resistance to environmental stresses, while lipid peroxidase activity was highest with the T-2 treatment, suggesting that this could lead to increased cellular membrane damage. In conclusion, high levels of biochemical components such as proline, catalaseand ascorbate peroxidase play a crucial role in the plant's ability to adapt to stress conditions and maintain healthy green growth. These levels can be optimized through the appropriate use of fertilizers. Fertilizer applications that support chlorophyll synthesis, enhance the plant's oxidative stress responseand increase resistance to environmental stresses contribute to both the physiological and biochemical resilience of the plant. By balancing these components, fertilizer use becomes a critical factor in optimizing plant health and ensuring successful green cover establishment.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent

the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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